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ORIGINAL ARTICLE

Peripheral blood stem cell versus bone marrow transplantation from HLA-identical sibling donors in patients with leukemia: a propensity score-based comparison from the Japan Society for Hematopoietic Stem Cell Transplantation registry

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Abstract We retrospectively analyzed the results of 707 adult patients who underwent myeloablative peripheral blood stem cell transplantation (PBSCT) ($n = 365$) and myeloablative bone marrow transplantation (BMT) ($n = 342$) for leukemia from HLA-identical sibling donors between 2000 and 2005 using the propensity score method. The results were obtained from the Japan Society for Hematopoietic Cell Transplantation registry. Multivariate Cox analysis showed that PBSCT was associated with lower overall survival (OS) in standard-risk patients [adjusted hazard ratio (aHR) = 1.83; 95% confidence interval (CI) 1.04–3.23; $P = 0.036$], but not in high-risk patients (aHR = 1.11; 95% CI 0.76–1.61; $P = 0.599$). Hematopoietic recovery was significantly faster after

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PBSCT. The risk of acquiring grade III–IV acute graft-versus-host disease (GVHD) (aHR = 2.23; $P = 0.040$) and extensive chronic GVHD (aHR = 1.93; $P = 0.001$) were significantly higher after PBSCT. PBSCT was associated with higher non-relapse mortality in standard-risk patients (aHR = 2.30; 95% CI 1.08–4.88; $P = 0.030$), but not in high-risk patients (aHR = 1.29; 95% CI 0.65–2.54; $P = 0.468$). Relapse after transplantation did not differ between PBSCT and BMT either in standard-risk group or in high-risk group (aHR = 1.17; 95% CI 0.55–2.52; $P = 0.684$ and aHR = 0.81; 95% CI 0.52–1.28; $P = 0.370$, respectively). In this retrospective analysis, OS was significantly lower after PBSCT in standard-risk patients, but not in high-risk patients. PBSCT was associated with significant risks of grade III–IV acute GVHD and extensive chronic GVHD.

Keywords Bone marrow transplantation · Peripheral blood stem cell transplantation · Allogeneic · Graft-versus-host disease

1 Introduction

During the past decade, allogeneic peripheral blood stem cell transplantation (allo-PBSCT) has been increasingly used as an alternative to allogeneic bone marrow transplantation (allo-BMT) [1]. Furthermore, allo-PBSCT is associated with rapid hematopoietic recovery. Several prospective randomized controlled trials conducted in Western countries have shown an increased incidence of chronic graft-versus-host disease (GVHD) [2–11]. Nevertheless, there is still substantial controversy regarding survival, acute GVHD, non-relapse mortality (NRM), and relapse [12–14].

Ethnicity has been reported to affect the incidence and severity of GVHD [15]. Japanese patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) were found to have a lower incidence of acute GVHD than those from Western patients [16, 17]. Therefore, the outcome of allo-PBSCT compared with that of allo-BMT may differ according to the ethnic background.

Using the propensity score method, we retrospectively analyzed the clinical outcomes of 707 adult Japanese leukemia patients who received allogeneic HSCT with myeloablative conditioning from HLA-identical sibling donors. These data were obtained from the Japan Society for Hematopoietic Cell Transplantation (JSHCT) registry. A propensity scoring system was devised to estimate the effects of treatments by comparing outcomes of those subjects who were not randomly assigned to experimental or control groups in an observational study [18]. A randomized control trial is superior in eliminating the confounding factors of known and unknown covariates by random treatment assignment. The propensity score expresses the likelihood of being assigned to experimental or control treatments, and is calculated using logistic regression models, including variables measured prior to treatment as much as possible. Considering the propensity score in this analysis, we expected that a hypothetical evaluation of an experimental trial in an observational study would give results similar to those of an evaluation in a randomized controlled trial.

2 Patients and methods

2.1 Study population

Using a standardized reporting form, JSHCT collects data on individual transplant patients from each transplant center, and follow-up reports are submitted annually after transplantation. A total of 1,426 patients, who underwent allogeneic HSCT between 2000 and 2005 for acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myelogenous leukemia (CML), have been reported to JSHCT. Patients were excluded from the study if their data were incomplete ($n = 205$), if they received a non-myeloablative or reduced-intensity conditioning regimen ($n = 223$), if they received grafts from other than HLA-identical siblings ($n = 217$), if they were less than 18 years of age ($n = 38$), if they had a previous history of HSCT ($n = 10$), and if they had non-allo-PBSCT or non-allo-BMT ($n = 16$). In Japan, most

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allo-HSCT patients have received granulocyte-colony stimulating factor (G-CSF) post-transplant [19]. The August 2006 data of the remaining 707 patients were analyzed. This study was approved by the Data Management Committee for the Nationwide Survey of JSHCT.

2.2 Definitions

Risk status at transplantation was categorized as either standard or high. Standard-risk diseases included acute leukemia in first complete remission (CR) and CML in first chronic phase (CP). Other disease status was categorized as high-risk disease [11]. The day of neutrophil engraftment was defined as the first of three consecutive days with an absolute neutrophil count (ANC) of more than 0.5×10^9/L. The day of platelet engraftment was defined as the first of seven consecutive days with a platelet count of more than 20×10^9/L without platelet transfusion. Acute GVHD was graded according to the standard criteria [20]. All patients who had no evidence of graft failure and survived beyond day 28 were considered to be evaluable for acute GVHD. GVHD persisting beyond day 100 and de novo GVHD occurring after day 100 were classified as chronic GVHD. The incidence of chronic GVHD was calculated in patients followed for more than 100 days, and the disease was classified as none, limited, or extensive [21]. Overall survival (OS) was defined as the duration of survival between transplantation and either death or the last follow-up.

Relapse was defined as disease progression with censored NRM. NRM included all causes of death other than relapse occurring at any time after transplantation. All deaths were considered in the estimation of OS.

2.2.1 *Endpoints*

The primary endpoint of comparison was OS. Secondary endpoints were hematopoietic recovery, acute GVHD (grade II–IV and III–IV), chronic GVHD (overall and extensive), NRM, and relapse.

2.2.2 *Propensity score calculation*

We calculated the propensity score using the pscore command in STATA version 10.1. (STATA, College Station, TX, USA) [22]. Factors included in the propensity score were as follows: age at HSCT in categories (<40, 40–49, and 50+) as an ordinal variable; sex (male/female) as an indicator variable; year of transplantation as a continuous variable; performance status at transplantation as an ordinal variable; risk status (CR1/CP1, CR2/CP2, or more advanced) as an indicator variable; a cumulative number of HSCT from related donors at an institution between 2000 and 2005 (1: 1–4, 62 institutions; 2: 5–11, 58 institutions; 3: 12 or more, 52 institutions) as an ordinal variable; and the percentage of allo-PBSCT out of total HSCT from HLA-identical siblings in tertile (1: <56%, 59 institutions; 2: 56–90%, 56 institutions; 3: 91% or more, 57 institutions) as an ordinal variable. We utilized as many variables as possible in the propensity score to evaluate the effects of known and unknown factors on the choice of treatment. After calculating the propensity score, the subjects were divided into four groups according to quartile. The numbers of subjects in quartiles 1–4 (allo-PBSCT/allo-BMT) were 23/154, 58/120, 126/50, and 158/18, respectively.

2.2.3 *Statistical analysis*

Patient characteristics and therapeutic outcomes were compared between allo-PBSCT and allo-BMT groups. OS was assessed using the Kaplan–Meier product limit method [23, 24]. Cumulative incidences of acute GVHD, chronic GVHD, NRM, and relapse were evaluated as 1 − (Kaplan–Meier estimate) instead of applying methods considering competing risks [25, 26] to maintain statistical consistency between logrank tests and methods of cumulative incidence estimation. Allo-PBSCT and allo-BMT groups were compared using the propensity score in quartiles [1–4], a stratified logrank test, and a stratified Cox proportional hazards model. Diagnosis (AML, ALL, and CML) and quartile of the propensity score were stratification factors. Confounders considered in the Cox proportional hazards model were as follows: year of diagnosis as a continuous variable; year of transplantation as a continuous variable; age at transplantation as a continuous variable; sex (male/female); sex matching (match/male to female/female to male/unknown); performance status (0, 1, 2, 3–4, and unknown); risk status (standard/high); GVHD prophylaxis [cyclosporin (CsA) + methotrexate (MTX), tacrolimus (TAC) + MTX, and others]; and conditioning regimen [total body irradiation (TBI)-containing regimen, busulfan and cyclophosphamide (BU/CY), and others]. All analyses were performed using STATA version 10.1, and P values less than 0.05 were considered statistically significant.

3 Results

3.1 Patient characteristics

The characteristics of patients are summarized in Table 1. The number of patients who underwent allo-PBSCT was 365, and that who underwent allo-BMT was 342. The median age at HSCT was 39 years (range 18–64 years) in the allo-PBSCT group and 39 years (range 18–59 years) in the allo-BMT group. The allo-PBSCT group included significantly more male patients from female donors than

Table 1 Characteristics of patients

	PBSCT *n* (%)	BMT *n* (%)	*P* value (Mann–Whitney test)
No. of patients	365	342	
Median patients age, years (range)	39, 18–64	39, 18–59	0.962
Patients sex (male/female)	210/155	189/153	0.543
Sex matching			
Matched	176 (48.2)	185 (54.1)	
Male to female	70 (19.2)	78 (22.8)	
Female to male	106 (29.0)	71 (20.8)	
Unknown	13 (3.6)	8 (2.3)	0.043
Risk group			
Standard-risk	149 (40.8)	202 (59.1)	
High-risk	216 (59.2)	140 (40.9)	<0.001
Diagnosis			
Standard-risk			
AML	58 (38.9)	76 (37.6)	
ALL	46 (30.9)	51 (25.2)	
CML	45 (30.2)	75 (37.2)	0.322
High-risk			
AML	128 (59.3)	75 (53.6)	
ALL	58 (26.9)	28 (20.0)	
CML	30 (13.8)	37 (26.4)	0.026
Performance status			
0	185 (50.7)	138 (40.4)	
1	73 (20.0)	55 (16.1)	
2	24 (6.6)	16 (4.7)	
3 or 4	12 (3.3)	2 (0.6)	
Unknown	71 (19.5)	131 (38.3)	<0.001
Conditioning regimen			
TBI-based	225 (61.6)	205 (59.9)	
Bu/CY	110 (30.1)	118 (34.5)	
Others	30 (8.3)	19 (5.6)	0.23
GVHD prophylaxis			
CsA + MTX	308 (84.4)	300 (87.7)	
TAC + MTX	12 (3.3)	14 (4.1)	
Others	45 (12.3)	28 (8.2)	0.176

Standard-risk diseases: acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase; other disease status was categorized as high-risk diseases

PBSCT peripheral blood stem cell transplantation, *BMT* bone marrow transplantation, *AML* acute myelogenous leukemia, *ALL* acute lymphoblastic leukemia, *CML* chronic myelogenous leukemia, *TBI* total body irradiation, *Bu* busulfan, *CY* cyclophosphamide, *GVHD* graft-versus-host disease, *CsA* cyclosporin, *MTX* methotrexate, *TAC* tacrolimus

the allo-BMT group (Mann–Whitney test, $P = 0.043$). AML, ALL, and CML were diagnosed in 337, 183, and 187 patients, respectively. The allo-PBSCT group included significantly more high-risk patients than the allo-BMT group ($P < 0.001$). Among the high-risk patients, the allo-BMT group had significantly more CML patients than the allo-PBSCT group ($P = 0.026$). Conditioning regimen and GVHD prophylaxis were performed according to the protocol of each institution, and there were no differences between the two groups. The most frequently used conditioning regimens were BU/CY (busulfan 1 mg/kg × 4/day × 4 days with cyclophosphamide 60 mg/kg/day × 2 days) and CY/TBI (cyclophosphamide 60 mg/kg/day × 2 days with total body irradiation 10–12 Gy). CsA plus MTX was used most frequently for GVHD prophylaxis. Median follow-up period for the surviving patients at the time of analysis was 33 months (1.8–55 months) in the PBSCT group and 31 months (1–53 months) in the BMT group.

3.2 Primary endpoint

3.2.1 Overall survival

Three-year OS in standard-risk patients was 68% [95% confidence interval (CI) 59–75] after allo-PBSCT and 77%

(95% CI 70–82) after allo-BMT (by disease and quartile in the propensity-score stratified logrank test; $P = 0.023$). Three-year OS in high-risk patients after allo-PBSCT and allo-BMT was 38% (95% CI 31–45) and 54% (95% CI 44–62), respectively ($P = 0.587$) (Fig. 1). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for lower OS in the population with standard-risk [adjusted hazard ratio (aHR) = 1.83; 95% CI 1.04–3.23; $P = 0.036$], but not that with high-risk (aHR = 1.11; 95% CI 0.76–1.61; $P = 0.599$).

3.3 Secondary endpoints

3.3.1 Hematopoietic recovery

Engraftment occurred in all patients receiving allo-PBSCT and allo-BMT (allo-PBSCT, $n = 324$; allo-BMT, $n = 305$) surviving for more than 28 days. Allo-PBSCT patients showed significantly faster neutrophil and platelet recovery compared with allo-BMT patients. The median time of recovery to ANC > 0.5×10^9/L was 14 days for the allo-PBSCT group and 16 days for the allo-BMT group, respectively (stratified logrank test, $P < 0.0001$). The median time of recovery to a platelet count >20×10^9/L was 15 days for the allo-PBSCT group and 21 days for the allo-BMT group, respectively ($P < 0.0001$). In the multivariate Cox analysis, allo-PBSCT was a significant factor for faster neutrophil (aHR = 0.57; 95% CI 0.45–0.71; $P < 0.001$) and platelet (aHR = 0.56; 95% CI 0.44–0.71; $P < 0.001$) recovery compared with allo-BMT.

3.3.2 Acute GVHD

The cumulative incidence of grade II–IV acute GVHD was 31% (95% CI 27–35) in all patients, whereas that in allo-PBSCT and allo-BMT groups was 35% (95% CI 30–41) and 26% (95% CI 22–32) (stratified logrank test, $P = 0.221$), respectively. The aHR for grade II–IV acute GVHD after allo-PBSCT was 1.25 (95% CI 0.85–1.84; $P = 0.260$) by multivariate Cox analysis. The cumulative incidence of grade III–IV acute GVHD was 14% (95% CI 10–18) and 5.4% (95% CI 3.3–8.8) in the allo-PBSCT and allo-BMT groups, respectively ($P = 0.021$). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for the development of grade III–IV acute GVHD (aHR = 2.23; 95% CI 1.04–4.78; $P = 0.040$; Fig. 2).

3.3.3 Chronic GVHD

The risk of chronic GVHD in the first year after transplantation was significantly higher after allo-PBSCT than after allo-BMT (cumulative incidence at 1 year, 51%; 95% CI 44–58 after allo-PBSCT vs. 34%; 95% CI 28–41 after allo-BMT; $P = 0.0005$ with stratified logrank test). The extensive form of chronic GVHD was more prevalent in the allo-PBSCT group than in the allo-BMT group (26%; 95% CI 21–33 with allo-PBSCT and 15%; 95% CI 11–20 with allo-BMT; $P = 0.0017$). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for the development of extensive chronic GVHD (aHR = 1.93; 95% CI 1.32–2.84; $P = 0.001$; Fig. 3).

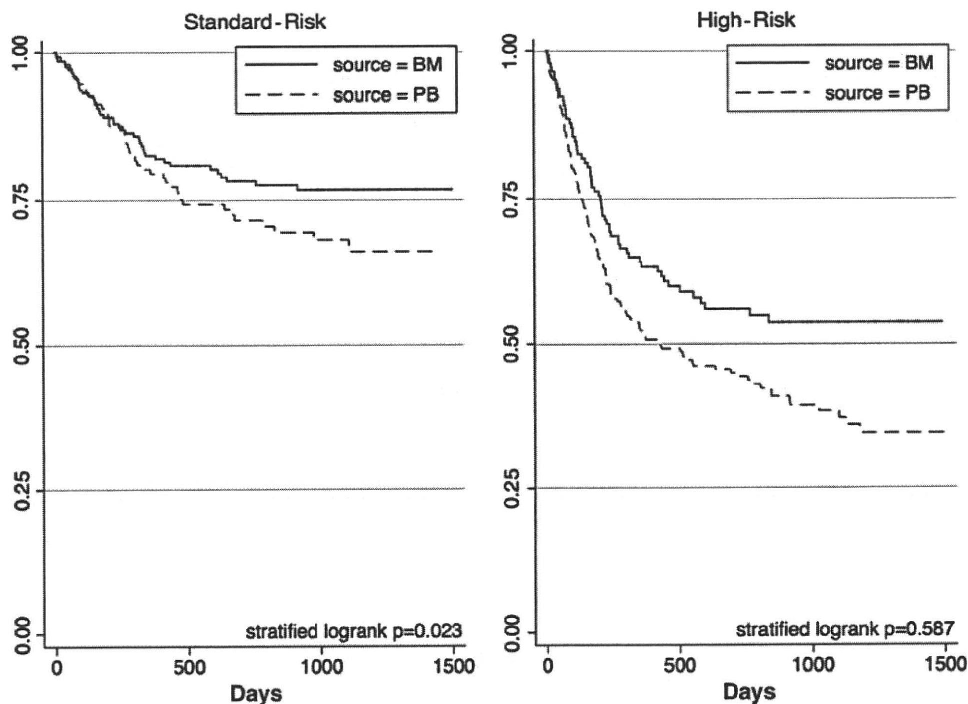

Fig. 1 Probabilities of overall survival after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

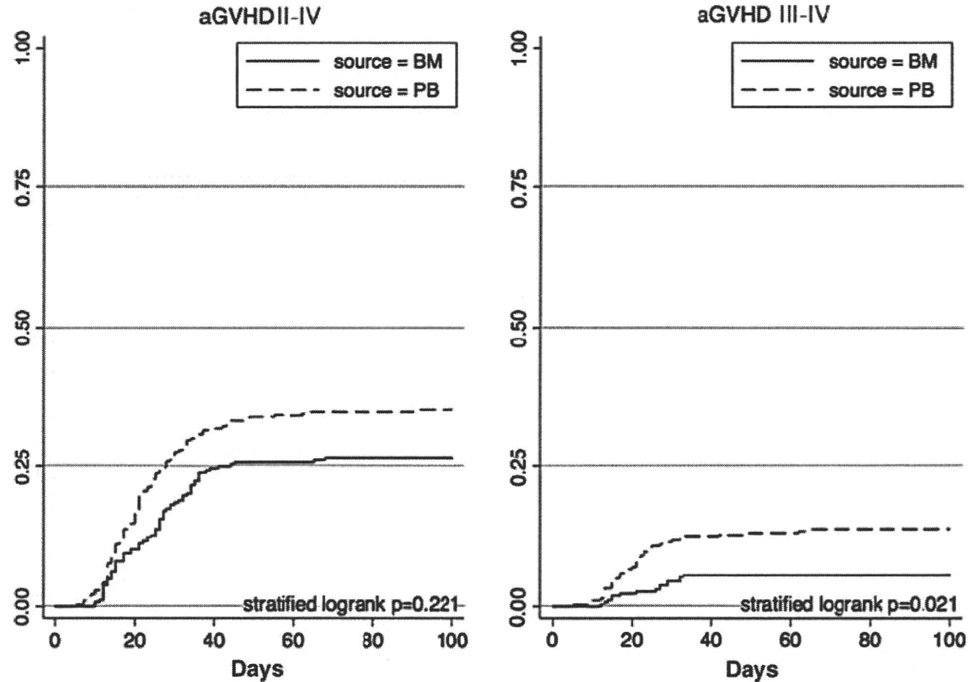

Fig. 2 Probabilities for grade II–IV and III–IV acute graft-versus-host disease (GVHD) after peripheral blood stem cell transplantation compared with bone marrow transplantation

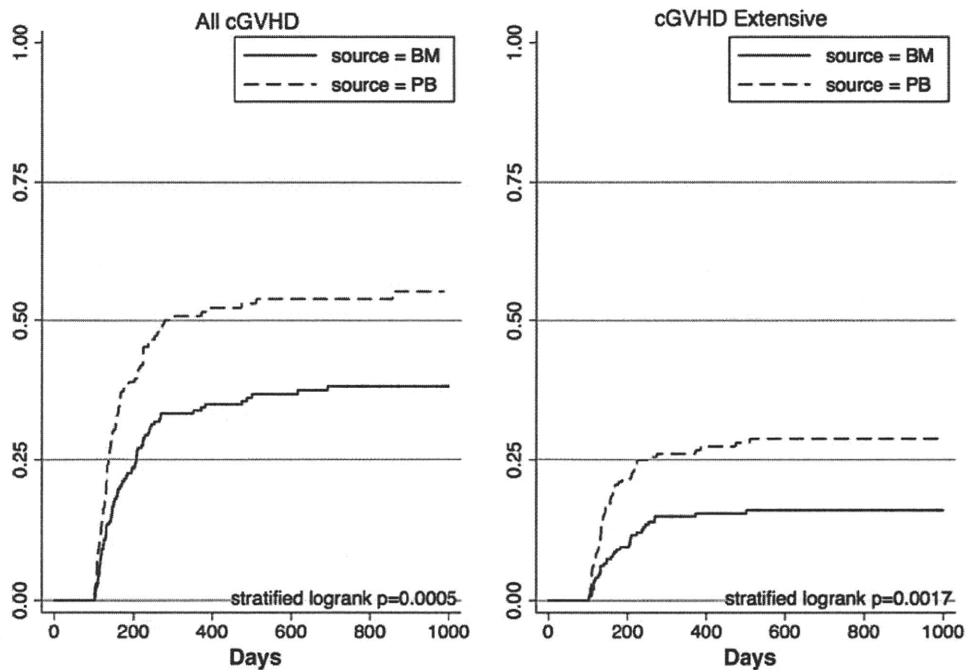

Fig. 3 Probabilities for chronic GVHD and extensive chronic GVHD after peripheral blood stem cell transplantation compared with bone marrow transplantation

3.3.4 Non-relapse mortality

The cumulative incidence of NRM for the standard-risk group at day 100 was 4.7% (95% CI 2.3–9.7) after allo-PBSCT and 6.0% (95% CI 3.4–10.2) after allo-BMT, and that at 1 year was 14.2% (95% CI 9.4–21.1) after allo-PBSCT and 11.2% (95% CI 8.0–17.2) after allo-BMT (stratified logrank test, $P = 0.047$). The cumulative incidence of NRM for the high-risk group at day 100 was 11.2% (95% CI 7.6–16.4) after allo-PBSCT and 8.9% (95% CI 5.1–15.1) after allo-BMT, and that at 1 year was 24.4% (95% CI 18.7–31.4) after allo-PBSCT and 14.7% (95% CI 9.6–22.2) after allo-BMT (stratified logrank test, $P = 0.221$) (Fig. 4).

Multivariate Cox analysis showed that allo-PBSCT was a significant factor for higher NRM in the standard-risk (aHR = 2.30; 95% CI 1.08–4.88; $P = 0.030$), but not in the high-risk (aHR = 1.29; 95% CI 0.65–2.54; $P = 0.468$).

3.3.5 Relapse

The cumulative incidence of relapse at 1 year for the standard-risk group was similar for allo-PBSCT (13.8%; 95% CI 8.9–21.0) and allo-BMT (9.7%; 95% CI 6.1–15.2) ($P = 0.518$ by stratified logrank test). Similarly, in the high-risk group the incidence was 32.4% (95% CI 25.6–40.3) for allo-PBSCT and 31.5% (95% CI 23.7–41.1) for allo-BMT ($P = 0.200$) (Fig. 5).

Multivariate Cox analysis showed no significant difference in the risk of relapse after allo-PBSCT and allo-BMT either in the standard-risk group or in the high-risk group (aHR = 1.17; 95% CI 0.55–2.52; $P = 0.684$ and aHR = 0.81; 95% CI 0.52–1.28; $P = 0.370$, respectively).

4 Discussion

In the present study, we analyzed results for 707 patients who underwent myeloablative HSCT for leukemia from HLA-identical sibling donors between 2000 and 2005. These data were obtained from the JSHCT registry. Health insurance coverage of allo-PBSCT was approved in Japan in 2000, and since then the number of allo-PBSCTs rapidly increased and exceeded the number of allo-BMTs between 2000 and 2003. Subsequently, the number of allo-PBSCTs decreased, and the numbers of allo-PBSCTs and allo-BMTs became equivalent in 2005. Thus, this analysis indicates the rather immature status of allo-PBSCT in Japan.

The Stem Cell Trialists' Collaborative Group [27] reported an individual patient data meta-analysis of nine randomized trials by comparing outcomes of allo-PBSCT versus allo-BMT from HLA-matched related donors for the treatment of hematologic malignancies. Allo-PBSCT was associated with a higher probability of 5-year OS in the subset analysis of patients with late disease due to decreased relapse. International Bone Marrow Transplant registry/European Group for Blood and Marrow Transplantation (IBMTR/EBMT) registry data of 398 adult allo-BMT and 208 allo-PBSCT patients with leukemia were analyzed using information on 6 or more years of follow-up [28]. OS in patients with early and advanced leukemia did not differ significantly between the two groups. The IBMTR report comparing outcomes after allo-PBSCT and allo-BMT for acute leukemia in children and adolescents showed that OS was lower after allo-PBSCT [29]. These controversial data indicate that the difference in stem cell source can affect OS depending on the underlying disease, disease status, and the patients' age.

In our study, OS was lower after allo-PBSCT than after allo-BMT in the standard-risk patients, but not in the high-risk patients. Considering the difference in stem cell source, factors affecting OS include hematopoietic and

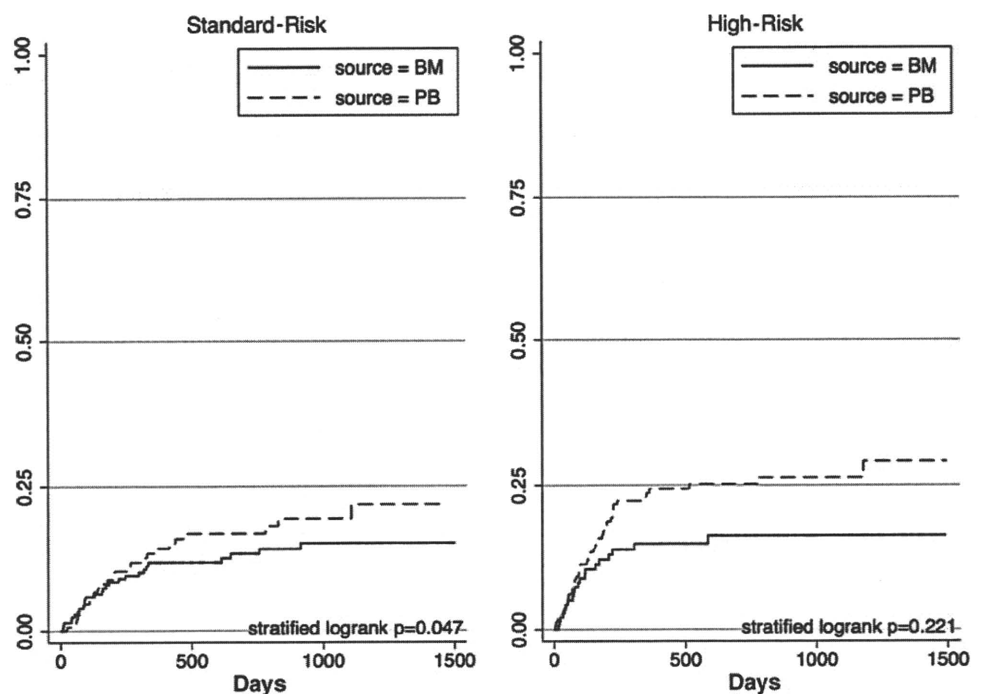

Fig. 4 Cumulative incidences of non-relapse mortality (NRM) after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

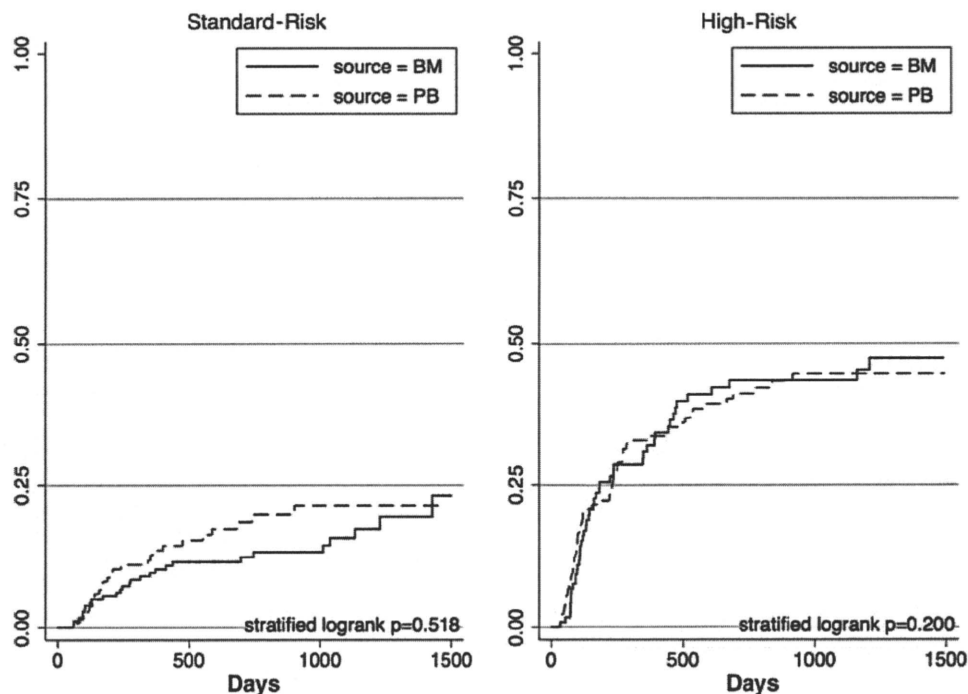

Fig. 5 Cumulative incidences of relapse after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

immune recovery, acute and chronic GVHD, and graft-versus-leukemia (GVL) effect or relapse [30].

In our analysis, allo-PBSCT was associated with more rapid hematopoietic recovery than allo-BMT as has been shown in most previous studies [4, 5, 11, 31]. Most randomized trials demonstrated that neutrophil recovery generally occurs 5–7 days earlier after allo-PBSCT compared with allo-BMT without G-CSF post-transplant [27, 32]. The EBMT study reported by Schmitz et al. [5] showed that neutrophil recovery was achieved 3 days earlier after allo-PBSCT than after allo-BMT with G-CSF post-transplant, and transplantation-related mortality did not differ between allo-PBSCT and allo-BMT groups. In Japan, most allo-HSCT patients receive G-CSF post-transplant, and in our study neutrophil recovery was observed 2 days earlier after allo-PBSCT than after allo-BMT. Accordingly, infectious complications may not decrease after allo-PBSCT compared to allo-BMT.

With regard to acute GVHD, the meta-analysis showed that allo-PBSCT was associated with a significant increase in the development of grade III–IV acute GVHD, but not grade II–IV acute GVHD [27]. In the present analysis, allo-PBSCT was also a significant factor in the incidence of grade III–IV acute GVHD. The increased incidence of grade III–IV acute GVHD in allo-PBSCT would have a negative effect on OS [33].

Extensive chronic GVHD was more frequent after allo-PBSCT than after allo-BMT in our study. This finding is in line with those of previous reports [5, 9, 11, 19, 31, 34].

In our analysis, NRM was higher after allo-PBSCT in the standard-risk patients, but not in the high-risk patients. The higher NRM after allo-PBSCT in the standard-risk group was likely due to increased grade III–IV acute GVHD and extensive chronic GVHD. Increased NRM after allo-PBSCT has been reported from children and adolescents suffering with acute leukemia [29]. A higher risk of mortality due to acute and chronic GVHD may counteract any benefit of more rapid hematopoietic recovery in the early transplant period.

In the allo-BMT setting, the development of both acute and chronic GVHD is associated with decreased relapse of leukemia, whereas the effect of GVHD on OS appears to be different depending on the study population [33, 35, 36]. The meta-analysis showed that allo-PBSCT was associated with a significant decrease in relapse in both early and late-stage disease patients [27]. On the contrary, increased extensive chronic GVHD in the allo-PBSCT group did not lead to a decrease in relapse in our analysis. We do not have a good explanation for this, but a similar observation was reported from the IBMTR/EBMT [28] registry data of adult patients with leukemia and the IBMTR [29] study in children and adolescents with acute leukemia. The advantage in term of the GVL effect with the cost of increased GVHD after allo-PBSCT relative to after allo-BMT remains controversial [27–29]. The allogeneic GVL effect varies from one disease to another, with the stage of the disease, and with donor histocompatibility. The GVL effect is believed to act while the leukemic burden is relatively

low [37]. Thus, to investigate the relationship between GVHD and relapse, subgroups differing in underlying disease and disease status would be needed.

We used the propensity score method to minimize selection bias. However, retrospective analysis has limitations. We could not exclude the possibility of unidentified confounding variables affecting the transplant outcomes and the inability to adjust the data for unknown or unmeasured factors. For example, we did not have data regarding pre-transplant infectious complications. Since allo-PBSCT is associated with more rapid hematopoietic recovery than allo-BMT, patients with serious infectious problems may have a tendency to undergo allo-PBSCT rather than allo-BMT. In this analysis, standard-risk diseases included acute leukemia in first CR and CML in first CP, while high-risk diseases included other diseases [11]. However, even in first CR acute leukemia patients, cytogenetic and molecular markers affect the prognosis with respect to survival in the allo-HSCT setting [38, 39]. We cannot deny the possibility that higher-risk patients in first CR tended to undergo allo-PBSCT. Thus, the results presented here should be interpreted with caution. It is also important to realize that our analysis was based on matched sibling myeloablative HSCT not on non-myeloablative HSCT. However, contrary to the result of the meta-analysis [27], multivariate Cox analysis showed that the allo-PBSCT group was associated with a lower OS in the populations with standard-risk. Prospective randomized trials are necessary to elucidate the advantages and disadvantages of allo-PBSCT in comparison with allo-BMT from HLA-identical sibling donors for the treatment of adult Japanese patients with leukemia.

Conflict of interest statement The authors declare no financial conflict of interest.

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Tyrosine Kinases Btk and Tec Regulate Osteoclast Differentiation by Linking RANK and ITAM Signals

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SUMMARY

Certain autoimmune diseases result in abnormal bone homeostasis, but association of immunodeficiency with bone is poorly understood. Osteoclasts, which derive from bone marrow cells, are under the control of the immune system. Differentiation of osteoclasts is mainly regulated by signaling pathways activated by RANK and immune receptors linked to ITAM-harboring adaptors. However, it is unclear how the two signals merge to cooperate in osteoclast differentiation. Here we report that mice lacking the tyrosine kinases Btk and Tec show severe osteopetrosis caused by a defect in bone resorption. RANK and ITAM signaling results in formation of a Btk(Tec)/BLNK(SLP-76)-containing complex and PLCγ-mediated activation of an essential calcium signal. Furthermore, Tec kinase inhibition reduces osteoclastic bone resorption in models of osteoporosis and inflammation-induced bone destruction. Thus, this study reveals the importance of the osteoclastogenic signaling complex composed of tyrosine kinases, which may provide the molecular basis for a new therapeutic strategy.

INTRODUCTION

Bone homeostasis depends on balanced action of bone-resorbing osteoclasts and bone-forming osteoblasts (Karsenty and Wagner, 2002). Tipping the balance in favor of osteoclasts leads to diseases with a low bone mass, whereas impaired osteoclastic bone resorption results in diseases with a high bone mass, including osteopetrosis (Teitelbaum and Ross, 2003). Bone reserves calcium and responds to calcium-regulating hormones, but osteoclasts and osteoblasts are not only regulated by the endocrine system. The immune and bone systems share numerous regulatory factors, including cytokines, receptors, signaling molecules, and transcription factors (Theill et al., 2002; Walsh et al., 2006; Takayanagi, 2007). Therefore, the pathology of one system may very well affect the other: it is well documented that enhanced bone resorption is associated with activation of the immune system observed in autoimmune or inflammatory diseases such as rheumatoid arthritis and periodontitis (Theill et al., 2002; Takayanagi, 2007).

Diseases with impaired immune responses are also associated with bone abnormalities, but it is poorly understood how the immunodeficiencies are functionally related to bone disorders. Hyperimmunoglobulin E (IgE) syndrome is characterized by skeletal symptoms such as osteoporosis (Kirchner et al., 1985) and scoliosis (Grimbacher et al., 1999), which are partly explained by the abnormality of osteoblasts and osteoclasts

caused by the mutation in the *STAT3* gene. (Minegishi et al., 2007). X-linked hyper-IgM syndrome (caused by a mutation in the *CD40L* gene) has a skeletal complication, osteopenia, which is attributed to enhanced osteoclast formation due to the impaired production of interferon-γ by T cells (Lopez-Granados et al., 2007). Thus, exploring the mechanisms underlying skeletal complications in primary immunodeficiencies will be important for understanding the shared mechanisms and crosstalk between immune and bone systems.

The osteoclast was the first skeletal cell to be determined to be under the control of the immune system (Horton et al., 1972). Because osteoclasts originate from bone marrow-derived monocyte/macrophage precursor cells (BMMs) of hematopoietic lineage, many regulators of osteoclast differentiation are also utilized in the control of the innate and adaptive immune systems. The differentiation of osteoclasts is mainly regulated by three signaling pathways activated by receptor activator of nuclear factor-κB ligand (RANKL), macrophage colony-stimulating factor (M-CSF), and immunoreceptor tyrosine-based activation motif (ITAM) (Asagiri and Takayanagi, 2007). Whereas M-CSF promotes the proliferation and survival of BMMs (Ross and Teitelbaum, 2005), RANKL activates the differentiation process by inducing the master transcription factor for osteoclastogenesis, nuclear factor of activated T cells c1 (NFATc1), via the tumor necrosis factor receptor-associated factor 6 (TRAF6) and c-Fos pathways (Takayanagi et al., 2002). The induction of NFATc1 is also dependent on the calcium signal, which is mediated by the activation of ITAM in adaptor molecules such as DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcRγ) (Koga et al., 2004; Mocsai et al., 2004) in association with costimulatory receptors of the immunoglobulin superfamily, including triggering receptor expressed in myeloid cells-2 (TREM-2), signal-regulatory protein β1 (SIRPβ1), paired immunoglobulin-like receptor-A (PIR-A), and osteoclast-associated receptor (OSCAR) (Koga et al., 2004). Phosphorylation of ITAM results in recruitment of the nonreceptor tyrosine kinase Syk, leading to the activation of phospholipase Cγ (PLCγ) and calcium mobilization (Faccio et al., 2003; Koga et al., 2004; Mocsai et al., 2004; Mao et al., 2006), but osteoclastogenesis cannot be induced by costimulatory signals alone and requires RANKL stimulation. However, it has been unclear how RANK and ITAM signals merge to cooperatively stimulate the downstream signaling pathway.

To identify the molecules activated by RANKL that stimulate the calcium signaling pathway by functioning as an integrator of the RANK and ITAM signals, we focused on nonreceptor tyrosine kinases because PLCγ initiates the calcium signal after tyrosine phosphorylation. Among the nonreceptor tyrosine kinases, genome-wide screening revealed Btk and Tec to be highly expressed in osteoclasts. The Tec kinase family, consisting of Bmx, Btk, Itk, Rlk, and Tec, is preferentially expressed in the hematopoietic system. T cell receptor (TCR) and B cell receptor (BCR) signaling complexes include Itk/Rlk and Tec/Btk, respectively (Schmidt et al., 2004). The role of Btk in antibody production is well recognized by the existence of immunodeficiencies, X-linked agammaglobulinemia (XLA) in humans (Tsukada et al., 1993) and X-linked immunodeficiency (Xid) in mice (Kerner et al., 1995), caused by a mutation in the *Btk* gene; in addition, mice with a combined deficiency of Tec family kinases display both unique and redundant functions in B cells (Btk and Tec) (Ellmeier et al., 2000) and T cells (Itk and Rlk) (Schaeffer et al., 1999). Here we report the crucial role of Btk and Tec in RANKL-induced osteoclastogenesis based on the genetic evidence obtained from $Tec^{-/-}Btk^{-/-}$ mice. In response to RANKL stimulation, Btk and Tec kinases form a signaling complex required for osteoclastogenesis with adaptor molecules such as BLNK, which also recruits Syk, thus linking the RANK and ITAM signals to phosphorylate PLCγ. This study provides a clear example of immunodeficiency association with abnormal bone homeostasis owing to defects in signaling molecules shared by B cells and osteoclasts. Moreover, therapeutic models suggest that suppression of Tec kinases can serve as a molecular basis for the development of future therapeutic strategies against bone diseases.

RESULTS

Osteoclasts Selectively Express Btk and Tec among Tec Kinases

RANK and ITAM signals cooperate to induce calcium signaling, but it has been unknown how these two signals are integrated. To gain insight into the molecules activated by RANKL that are potentially involved in the activation of tyrosine phosphorylation of the PLCγ pathway, we performed a genome-wide screening of mRNAs for nonreceptor tyrosine kinases expressed during osteoclast and osteoblast differentiation (Figure 1A). We identified high expression of *Src* and *Syk*, whose crucial functions in the osteoclast lineage have been well documented (Soriano et al., 1991; Faccio et al., 2003; Koga et al., 2004). Interestingly, osteoclasts, but not osteoblasts, express the mRNAs for *Btk* and *Tec* at a higher level than that for *Src* (Figure 1A). The selective expression of Btk and Tec in osteoclast precursor cells was also confirmed by RT-PCR (Figure 1B) and immunoblot analysis (Figure 1C).

Osteopetrotic Phenotype of Mice Doubly Deficient in Btk and Tec

The results led us to analyze the bone phenotype of mice deficient in Tec (Ellmeier et al., 2000), Btk (Kerner et al., 1995), or both (Ellmeier et al., 2000). Microradiological analysis revealed the trabecular bone volume to be normal in $Tec^{-/-}$ mice, minimally increased in $Btk^{-/-}$ mice, and markedly increased in $Tec^{-/-}Btk^{-/-}$ mice (Figure 1D). Microcomputed tomography clearly indicated that the bone volume was greatly enhanced in $Tec^{-/-}Btk^{-/-}$ mice (Figure 1E). Histological analysis revealed the osteoclast number was significantly reduced in the epiphyseal region, and the bone marrow was abnormally filled with trabecular bone in $Tec^{-/-}Btk^{-/-}$ mice (Figure 1F). We also observed cartilage remnants characteristic of osteopetrosis in $Tec^{-/-}Btk^{-/-}$ mice (Figure 1F). Bone morphometric analysis indicated an increase in bone volume associated with a reduced osteoclast number, a decrease in the indicators of osteoclastic bone resorption (Figure 1G), and a normal level of bone formation in $Tec^{-/-}Btk^{-/-}$ mice (Figure 1H). There was no significant difference in the resorption and formation parameters among the wild-type (WT), $Tec^{-/-}$, and $Btk^{-/-}$ mice (see Figure S1 available online). These results collectively suggest that the increase in bone mass in $Tec^{-/-}Btk^{-/-}$ mice is caused by impaired osteoclastic bone resorption owing to a defect in osteoclast differentiation.

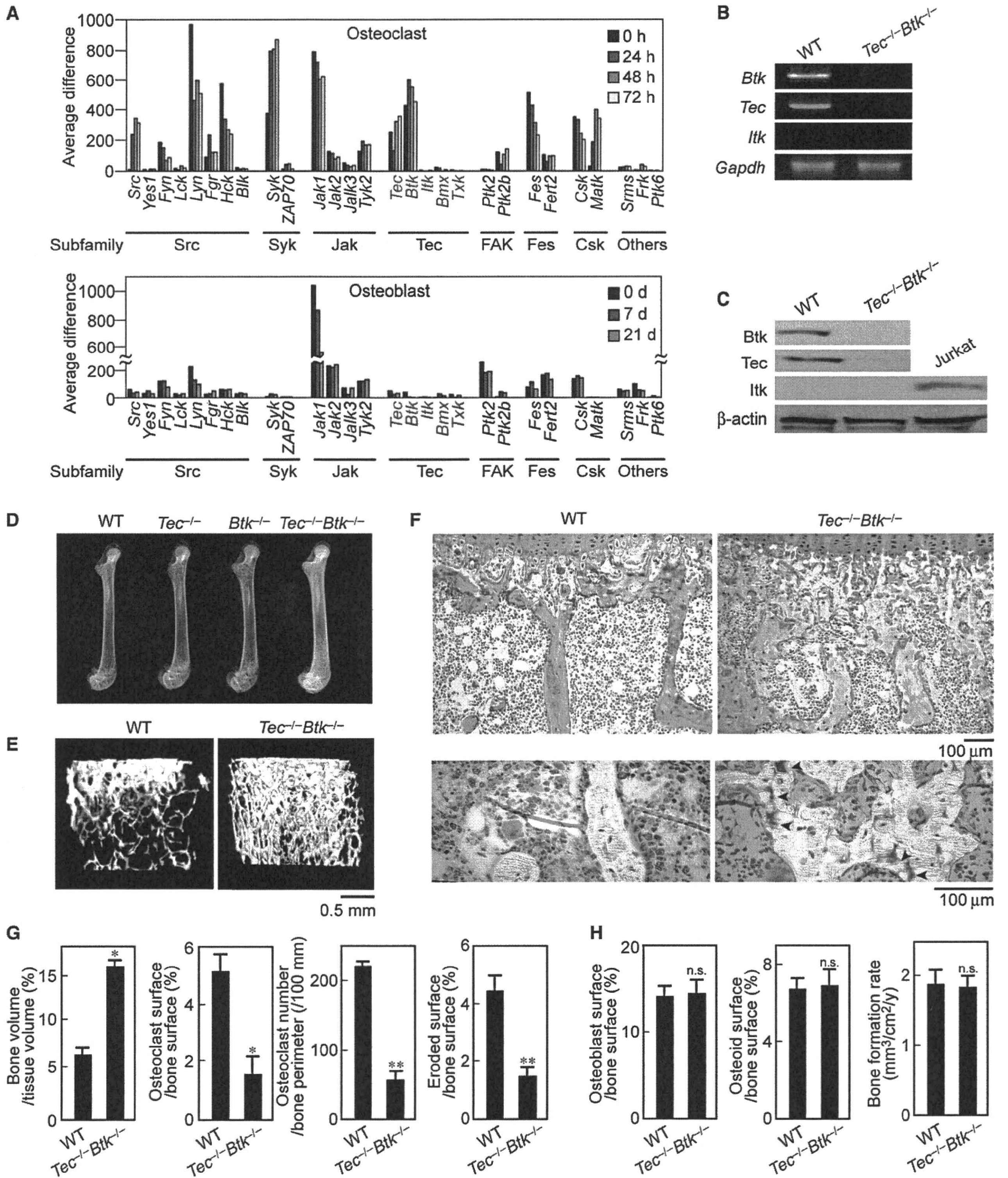


Figure 1. Osteopetrotic Phenotype of *Tec*$^{-/-}$*Btk*$^{-/-}$ Mice

(A) GeneChip analysis of mRNAs for nonreceptor tyrosine kinases during osteoclast and osteoblast differentiation. FAK, focal adhesion kinase; Fes, feline sarcoma oncogene; Csk, c-src tyrosine kinase.

(B) RT-PCR analysis of *Tec*, *Btk*, and *Itk* mRNAs in WT and *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs.

(C) Expression of Tec, Btk, and Itk proteins in WT and *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs.

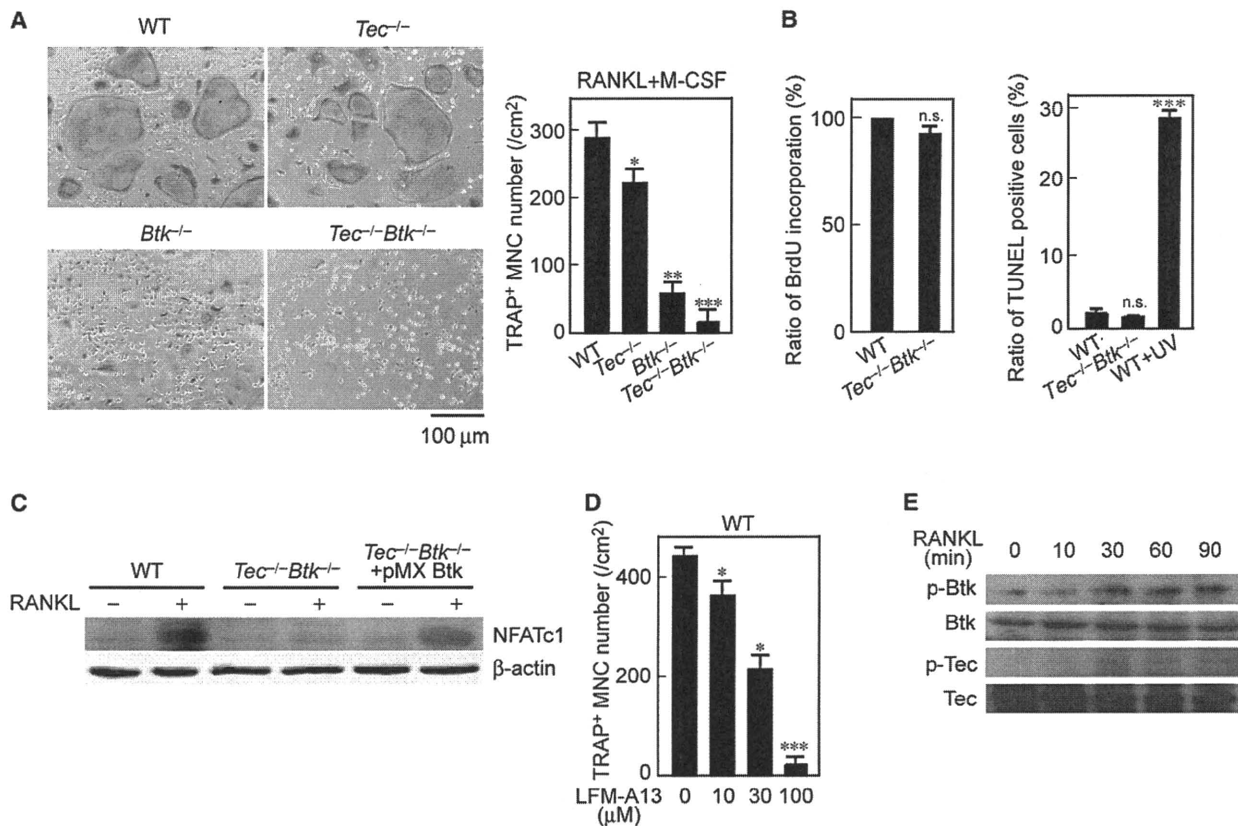


Figure 2. Crucial Role of Tec Family Kinases in RANKL-Induced Osteoclast Differentiation

(A) Osteoclast differentiation from WT, $Tec^{-/-}$, $Btk^{-/-}$, and $Tec^{-/-}Btk^{-/-}$ BMMs in response to RANKL and M-CSF.

(B) Cell proliferation rate (BrdU incorporation assay) and apoptosis (TUNEL assay) of WT and $Tec^{-/-}Btk^{-/-}$ BMMs stimulated with RANKL and M-CSF.

(C) NFATc1 induction in WT and $Tec^{-/-}Btk^{-/-}$ BMMs 72 hr after RANKL stimulation. Retroviral introduction of Btk into $Tec^{-/-}Btk^{-/-}$ BMMs recovers the NFATc1 induction.

(D) Effect of the Tec kinase inhibitor LFM-A13 on the osteoclast differentiation induced by RANKL and M-CSF.

(E) Phosphorylation of Btk and Tec in BMMs in response to RANKL.

Despite the severe defect in osteoclastic bone resorption, the $Tec^{-/-}Btk^{-/-}$ mice had normal tooth eruption, but the results nevertheless suggest that the two kinases play a critically important role in osteoclast differentiation.

Btk and Tec Are Activated by RANKL and Indispensable for Osteoclastogenesis

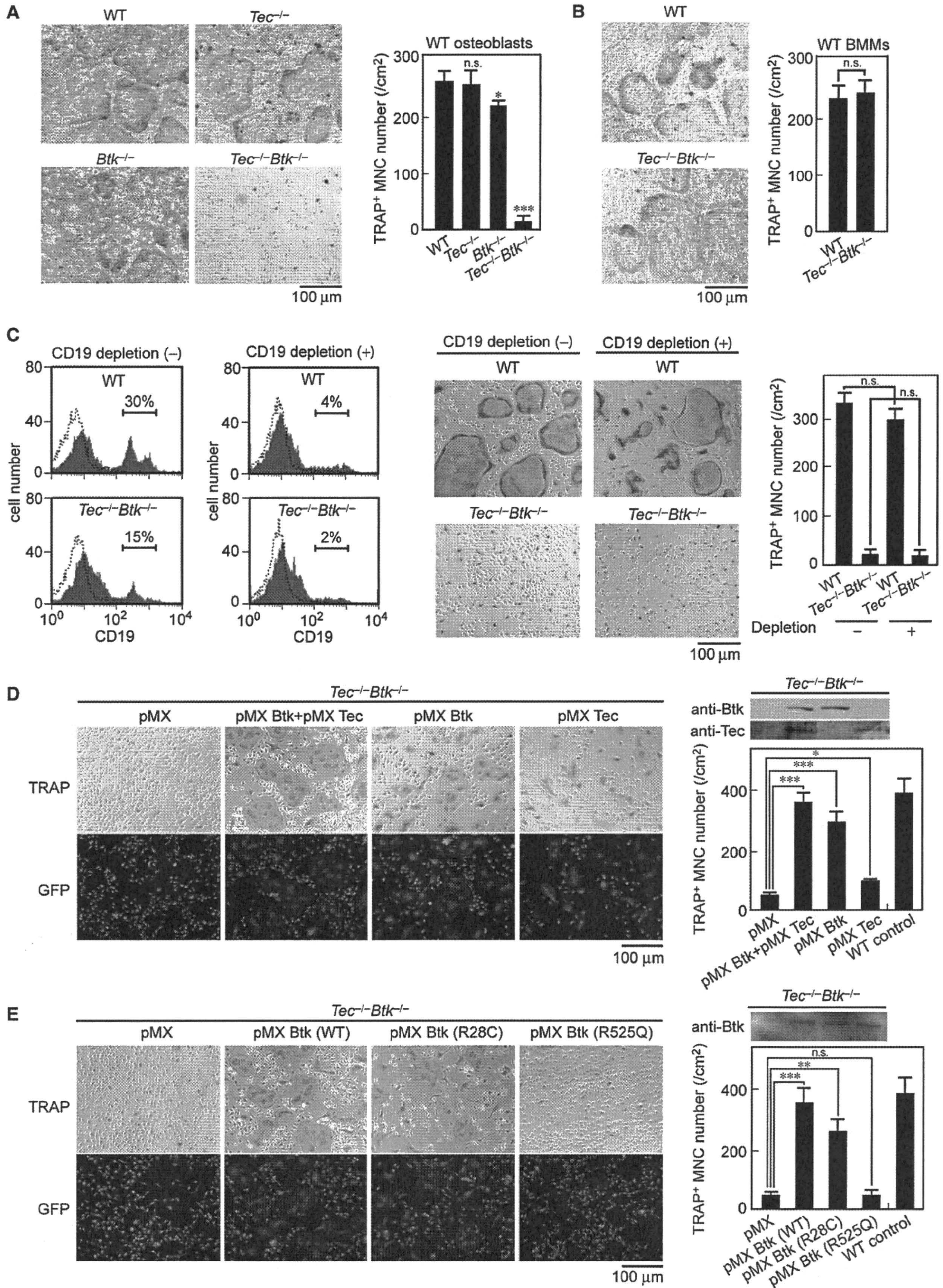
In vitro osteoclast differentiation was evaluated by counting the multinucleated cells (MNCs) positive for the osteoclast marker tartrate-resistant acid phosphatase (TRAP) after stimulation of BMMs with recombinant RANKL in the presence of M-CSF. Osteoclast differentiation was only minimally affected in $Tec^{-/-}$ cells but was severely impaired in $Btk^{-/-}$ cells and almost completely abrogated in $Tec^{-/-}Btk^{-/-}$ cells (Figure 2A). There was no significant difference in M-CSF-dependent proliferation of precursor cells or the rate of apoptosis between WT and $Tec^{-/-}Btk^{-/-}$ cells (Figure 2B) or in the number of CD11b^{+} cells in the M-CSF-stimulated bone marrow cells (data not shown). Therefore, there is no defect in the generation of osteoclast precursor cells in $Tec^{-/-}Btk^{-/-}$ mice, although we observed that the survival of mature macrophages derived from $Tec^{-/-}Btk^{-/-}$ mice was partly impaired when cultured with a low concentration of M-CSF (W.E., unpublished data). RANKL-stimulated induction of NFATc1, the key transcription factor for osteoclastogenesis, was severely suppressed in $Tec^{-/-}Btk^{-/-}$ cells (Figure 2C) and $Btk^{-/-}$ cells (data not shown). We also analyzed the effect of the Tec kinase inhibitor LFM-A13 (Mahajan et al., 1999), which was designed to specifically bind the ATP-binding site of Btk,

(D) Microradiograph of the femur of WT, $Tec^{-/-}$, $Btk^{-/-}$, and $Tec^{-/-}Btk^{-/-}$ mice.

(E) Microcomputed tomography of the epiphyseal region of the femurs of WT and $Tec^{-/-}Btk^{-/-}$ mice.

(F) Histological analysis of the proximal tibiae of WT and $Tec^{-/-}Btk^{-/-}$ mice (upper, TRAP/hematoxylin; lower, toluidine blue stainings). Note that the number of TRAP-positive cells is markedly decreased in $Tec^{-/-}Btk^{-/-}$ mice. The bone marrow is filled with unresorbed bone, in which cartilage remnants (arrowheads) characteristic of osteopetrosis are observed.

(G and H) (G) Bone volume and parameters for osteoclastic bone resorption and (H) parameters for osteoblastic bone formation in the bone morphometric analysis of WT and $Tec^{-/-}Btk^{-/-}$ mice. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; n.s., not significant (throughout the paper).


A
WT
Tec−/−
Btk−/−
Tec−/−Btk−/−
100 μm
WT osteoblasts
n.s.
TRAP+ MNC number (/cm2)
WT
Tec−/−
Btk−/−
Tec−/−Btk−/−
B
WT
Tec−/−Btk−/−
100 μm
WT BMMs
n.s.
TRAP+ MNC number (/cm2)
C
CD19 depletion (−)
CD19 depletion (+)
WT
30%
4%
Tec−/−Btk−/−
15%
2%
cell number
CD19
100 μm
TRAP+ MNC number (/cm2)
Depletion
D
Tec−/−Btk−/−
pMX
pMX Btk+pMX Tec
pMX Btk
pMX Tec
TRAP
GFP
100 μm
anti-Btk
anti-Tec
WT control
E
pMX Btk (WT)
pMX Btk (R28C)
pMX Btk (R525Q)

and which was revealed subsequently to suppress other Tec kinases additionally (Fernandes et al., 2005), on osteoclast differentiation. LFM-A13 strongly inhibited RANKL-induced osteoclastogenesis (Figure 2D) and NFATc1 induction (data not shown). In contrast, this compound only slightly affected the bone resorption activity of osteoclasts (Figure S2). Importantly, phosphorylation of Btk and Tec was induced by RANKL stimulation in BMMs (Figure 2E). Thus, the tyrosine kinases Btk and Tec are activated by RANKL and play a crucial role in RANKL-induced signal transduction during osteoclastogenesis.

Impaired Osteoclastogenesis in *Tec*$^{-/-}$*Btk*$^{-/-}$ Mice Caused by a Cell-Autonomous Mechanism

To determine whether impaired osteoclastogenesis in *Tec*$^{-/-}$*Btk*$^{-/-}$ mice is caused by a cell-autonomous mechanism, we evaluated the osteoclastogenesis in the coculture system of bone marrow cells with osteoblasts. In the coculture of bone marrow cells derived from mutant mice with WT osteoblasts, osteoclast formation was not severely affected in *Tec*$^{-/-}$ or *Btk*$^{-/-}$ cells but was almost abrogated in *Tec*$^{-/-}$*Btk*$^{-/-}$ cells (Figure 3A). In contrast, *Tec*$^{-/-}$*Btk*$^{-/-}$ osteoblasts were normally able to support osteoclastogenesis of WT bone marrow cells (Figure 3B). These results suggest that the impaired osteoclastogenesis in *Tec*$^{-/-}$*Btk*$^{-/-}$ mice is caused by a defect in osteoclast precursor cells. Consistent with the observation that the *Btk*$^{-/-}$ mice have a normal number of osteoclasts in vivo (Figure S1), *Btk*$^{-/-}$ cells differentiate into osteoclasts in the coculture system essentially in a normal manner (Figure 3A), indicating that the larger contribution of Btk is observed only under limited in vitro conditions, including the RANKL/M-CSF-stimulated BMM culture system (Figure 2A). Since a similar observation has been made in *DAP12*$^{-/-}$ cells (Koga et al., 2004), we infer that Btk is more closely associated with the DAP12-dependent pathway.

Since *Tec*$^{-/-}$*Btk*$^{-/-}$ mice have almost no B cells and exhibit severe immunodeficiency (but their T cells are not affected) (Ellmeier et al., 2000), it is possible that the impaired osteoclastogenesis is influenced by differences in the B cell number in the bone marrow. Therefore, we examined whether depletion or addition of CD19$^+$ B cells had any effect on osteoclastogenesis from bone marrow cells containing B cells. Osteoclast differentiation was not influenced by the coexistence of B cells in either WT or *Tec*$^{-/-}$*Btk*$^{-/-}$ cells (Figure 3C). Taken together, these results indicate that the impaired osteoclastogenesis in *Tec*$^{-/-}$*Btk*$^{-/-}$ mice is not related to an abnormality of other cell types such as osteoblasts or B cells, supporting the notion that the impaired osteoclast differentiation is caused by a cell-autonomous mechanism.

Consistent with this notion, retrovirus-mediated expression of Btk and Tec almost completely recovered the osteoclast differentiation in *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs. The introduction of Btk into *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs by retroviral transfer markedly rescued the differentiation blockade, but that of Tec rescued it to a lesser extent (Figure 3D). This result is consistent with the dominant role of Btk in osteoclastogenesis in the BMM culture system (Figure 2A). Tec family kinases contain a pleckstrin-homology (PH) domain that binds to phosphatidylinositol-3,4,5-triphosphate (PtdIns[3,4,5]P_3), a lipid product of phosphoinositide 3-kinase, a Tec homology domain, and a carboxyl-terminal kinase domain (Schmidt et al., 2004). A kinase-inactive mutant of Btk (R525Q) that carries a mutation in the kinase domain (Takata and Kurosaki, 1996) did not rescue the osteoclast differentiation blockade, but another mutant (R28C) that harbors a mutation in the PH domain (Takata and Kurosaki, 1996), which causes a certain type of XLA in humans (Tsukada et al., 1993), exhibited a capacity for rescue comparable to the WT Btk (Figure 3E). The results suggest that the function of Btk in osteoclastogenesis requires its kinase activity but does not completely depend on the interaction with PtdIns(3,4,5)P_3 through the PH domain.

Tec Kinases Link the RANK Signal to the Phosphorylation of PLCγ

How does deficiency of Btk and Tec affect the osteoclastogenic signaling pathways? To address these issues by a systems biology approach, we utilized a systematic protein-protein interaction database created by the Genome Network Project (http://genomenetwork.nig.ac.jp/index.html), in which our laboratory has served as a working member. From this database, we extracted the protein-protein interaction network related to the two major signaling pathways regulating osteoclastogenesis, the RANK and ITAM pathways, with which we merged our original data on the increase in mRNA expression after RANKL stimulation (Figure 4A). The RANK-associated gene network included TRAF6, NF-κB, and mitogen-activated protein kinase (MAPK), whereas the ITAM (DAP12/FcRγ)-associated gene network included Syk, PLCγ, and Btk/Tec. As expected, the expression of many RANK-associated molecules was upregulated by RANKL stimulation. Interestingly, the expression of many of the genes in the ITAM-associated gene network was also upregulated by RANKL stimulation.

Based on these results, together with the observation that Btk and Tec were activated by RANKL (Figure 2E), we hypothesized that Btk and Tec are the molecules that bridge the RANK and ITAM pathways to activate calcium signaling. Notably, RANKL-induced tyrosine phosphorylation of PLCγ1 and PLCγ2 was markedly suppressed in *Tec*$^{-/-}$*Btk*$^{-/-}$ cells (Figure 4B). Furthermore, the RANKL-induced calcium oscillation required for *NFATc1* induction was barely observed in *Tec*$^{-/-}$*Btk*$^{-/-}$ cells (Figure 4C). In contrast, we observed a normal level of RANKL-induced activation of MAPKs (ERK, JNK, p38), IκB kinase α (IKKα),

Figure 3. Impaired Osteoclastogenesis in *Tec*$^{-/-}$*Btk*$^{-/-}$ Mice Caused by a Cell-Autonomous Mechanism

(A) Osteoclast differentiation from WT, *Tec*$^{-/-}$, *Btk*$^{-/-}$, and *Tec*$^{-/-}$*Btk*$^{-/-}$ bone marrow cells in the coculture system with WT osteoblasts.

(B) Osteoclast differentiation from WT bone marrow cells in the coculture system with WT or *Tec*$^{-/-}$*Btk*$^{-/-}$ osteoblasts.

(C) Effect of CD19$^+$ B cell depletion on osteoclast differentiation. The number of CD19$^+$ cells before and after depletion of CD19$^+$ B cells in bone marrow cells derived from WT or *Tec*$^{-/-}$*Btk*$^{-/-}$ mice (left). Osteoclast differentiation from WT and *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs after depletion of CD19$^+$ B cells in response to RANKL and M-CSF (right).

(D) Effects of retroviral expression of Tec (pMX Tec) and/or Btk (pMX Btk) on osteoclastogenesis from *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs stimulated with RANKL and M-CSF.

(E) Effects of retroviral expression of WT Btk or Btk mutants harboring a mutation in the PH domain (R28C) or the kinase domain (R525Q) on osteoclastogenesis from *Tec*$^{-/-}$*Btk*$^{-/-}$ BMMs stimulated with RANKL and M-CSF.

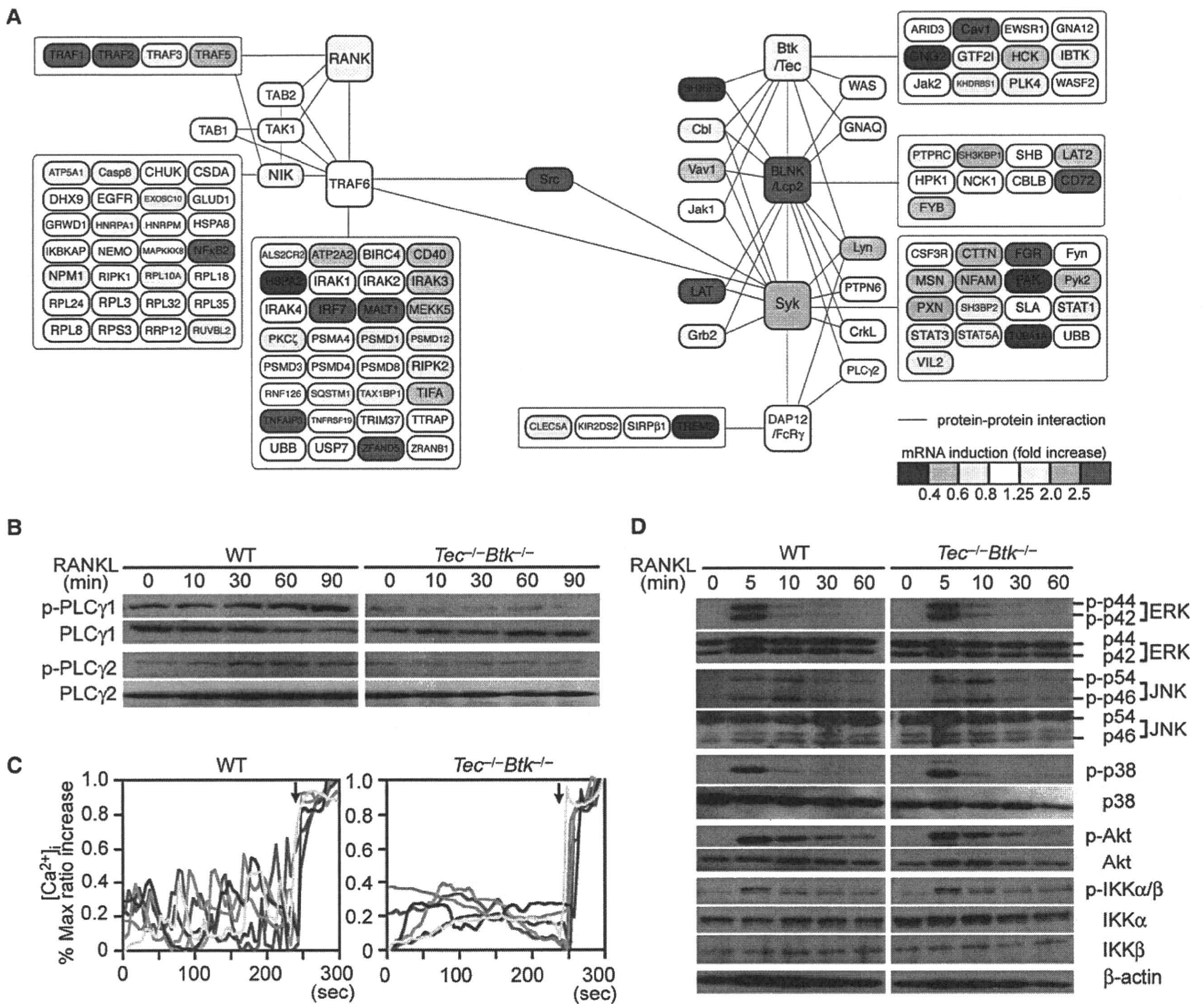


Figure 4. Tec Kinases Mediate Osteoclastogenic Signaling Pathway

(A) Dynamic protein-protein interaction network in osteoclast differentiation. Proteins connected with a black line are interacting partners, and the color indicates how many fold mRNA expression was increased 24 hr after RANKL stimulation.

(B) RANKL-induced PLCγ1 and PLCγ2 phosphorylation in WT and $Tec^{-/-}Btk^{-/-}$ BMMs.

(C) Oscillatory change in the intracellular Ca^{2+} concentration in WT and $Tec^{-/-}Btk^{-/-}$ BMMs after RANKL stimulation. The addition of 10 μM ionomycin at the end of each experiment is indicated by an arrow. Each color indicates a different cell in the same field.

(D) RANKL-induced ERK, JNK, p38, Akt, and IKK phosphorylation in WT and $Tec^{-/-}Btk^{-/-}$ BMMs.

IKKβ, and Akt, all of which are activated downstream of TRAF6 (Wong et al., 1999) (Figure 4D). These results indicate that RANKL-activated Btk and Tec are selectively involved in the phosphorylation of PLCγ, and a defect in calcium signaling causes the impaired *NFATc1* induction in $Tec^{-/-}Btk^{-/-}$ cells. Thus, Btk and Tec link the RANK signal to calcium signaling in the osteoclast lineage.

Tec Kinases Form an Osteoclastogenic Signaling Complex with Scaffold Proteins

Since PLCγ activation is known to be dependent on the ITAM signal, another question arises as to how PLCγ activation is regulated by both RANK and ITAM signals. The ITAM-associated gene network indicates that ITAM associates with Syk, which associates with and phosphorylates scaffold proteins such as BLNK (Ishiai et al., 1999). Because BLNK associates with Btk in a phosphorylation-dependent manner in B cells (Hashimoto et al., 1999), we examined whether RANKL-activated Btk is recruited to BLNK in BMMs. The colocalization of Btk and BLNK was increased, at what appeared to be the plasma membrane, after RANKL stimulation (Figure 5A and Figure S3). This translocation was not observed in $DAP12^{-/-}FcR\gamma^{-/-}$ cells (Figure 5A and Figure S3), suggesting that the ITAM signals are also required for the formation of the Btk-BLNK complex. Consistent with this, immunoblot analysis showed that Btk coimmunoprecipitated with BLNK in BMMs in the presence of RANKL, but

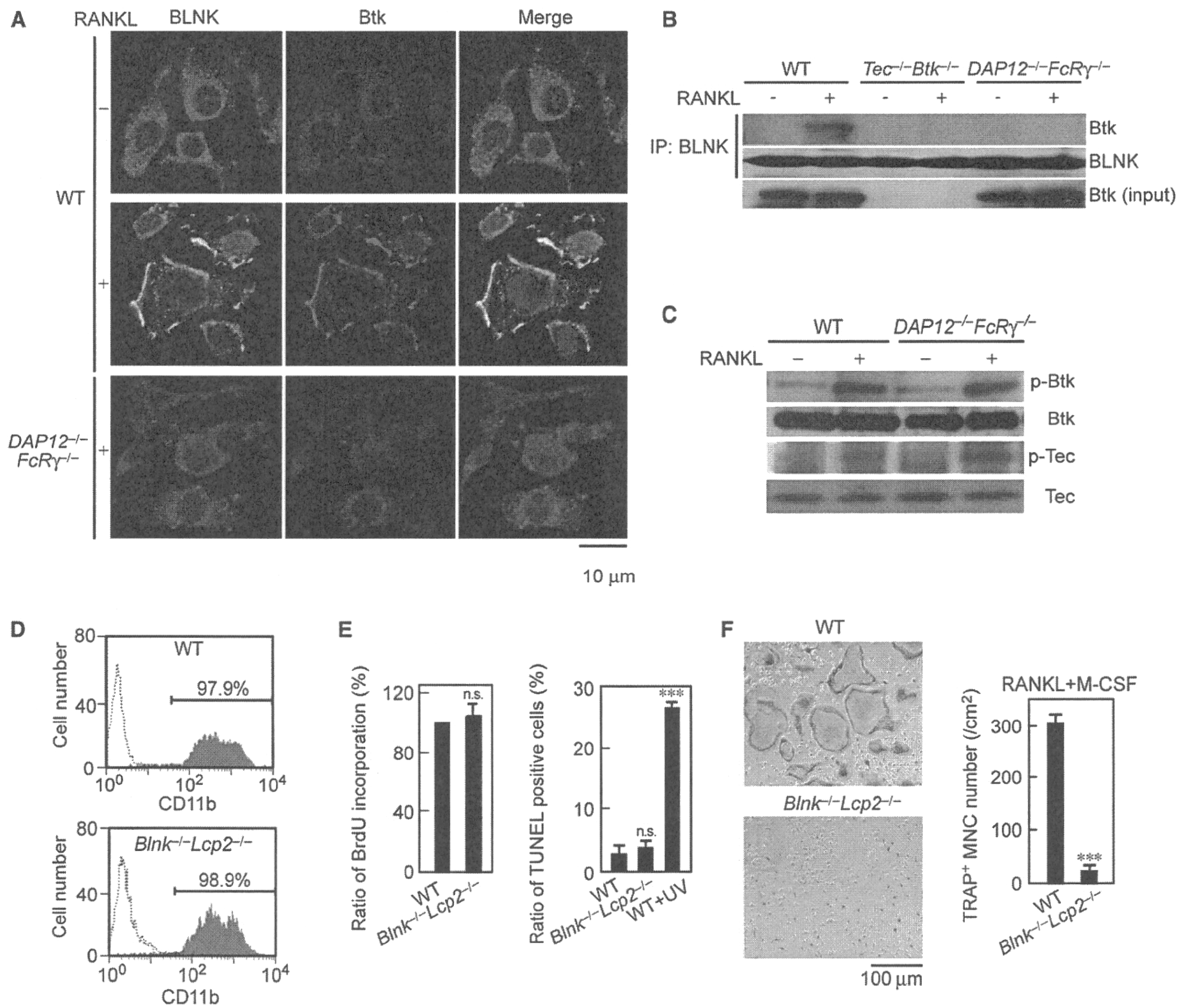


Figure 5. Tec Kinases Form an Osteoclastogenic Signaling Complex with Scaffold Proteins

(A) Immunofluorescence microscopic analysis of colocalization of Btk and BLNK in WT and *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ cells 15 min after RANKL stimulation. The nuclei were stained with Hoechst 33342.

(B) Immunoprecipitation analysis of the interaction between Btk and BLNK in WT and *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ cells 15 min after RANKL stimulation.

(C) Tyrosine phosphorylation of Tec kinases in WT and *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ cells 15 min after RANKL stimulation.

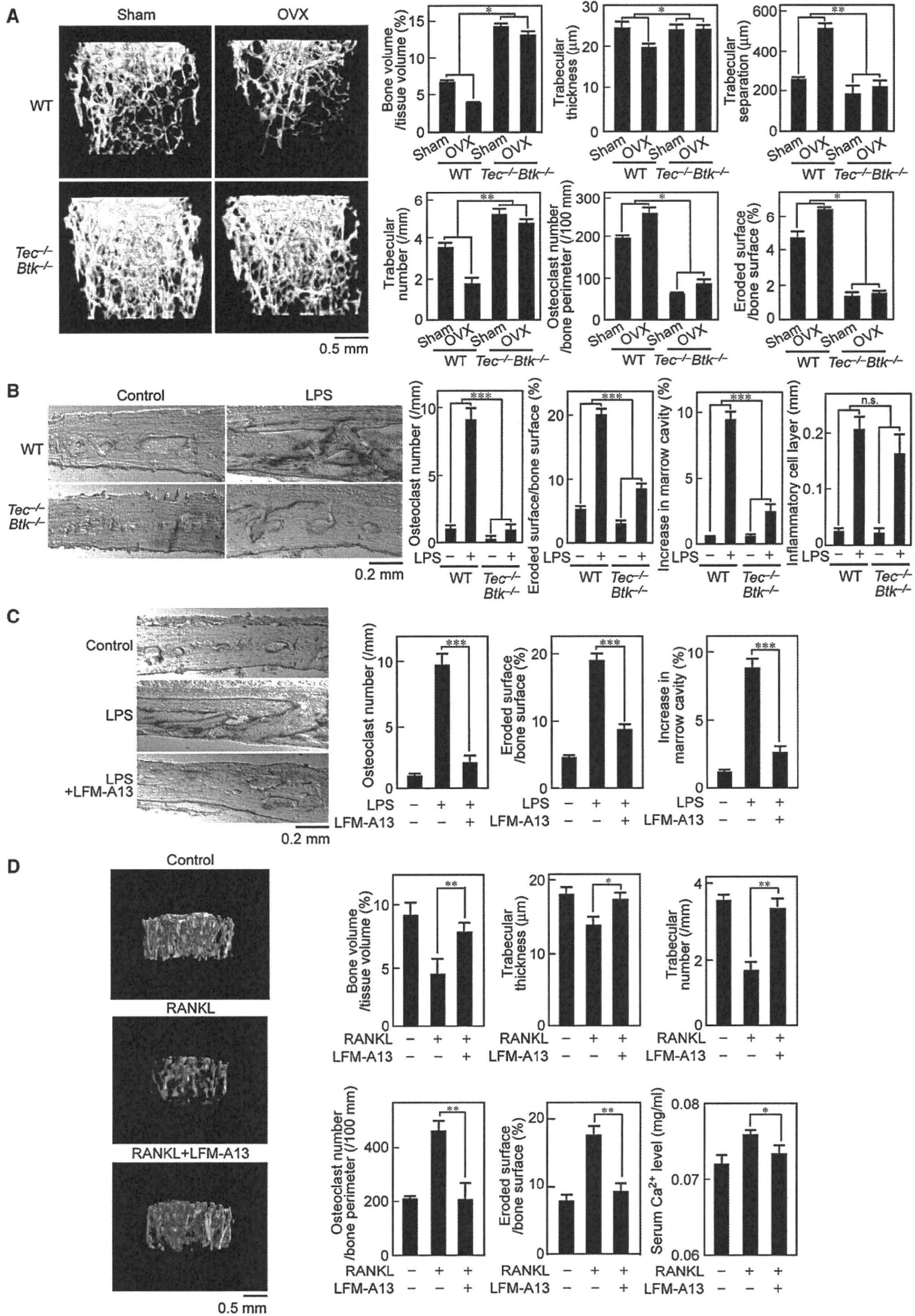
(D) Expression of CD11b in the M-CSF-stimulated bone marrow cells from WT and *Blnk*$^{-/-}$*Lcp2*$^{-/-}$ mice (flow cytometry).

(E) Cell proliferation rate (BrdU incorporation assay) and apoptosis (TUNEL assay) of WT and *Blnk*$^{-/-}$*Lcp2*$^{-/-}$ BMMs stimulated with RANKL and M-CSF.

(F) Osteoclast differentiation from WT and *Blnk*$^{-/-}$*Lcp2*$^{-/-}$ BMMs in response to RANKL and M-CSF.

the association was abrogated in *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ cells (Figure 5B). Furthermore, Tec kinases were phosphorylated in response to RANKL, even in *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ cells (Figure 5C). Thus, the activation of Btk by RANK and the activation of BLNK by the ITAM signal are both required for the association of Btk with BLNK, which may function as the molecular switch that integrates RANK and ITAM signals.

To further investigate the role of BLNK in osteoclastogenesis, we analyzed osteoclast differentiation in BMMs derived from *Blnk*$^{-/-}$ mice (Hayashi et al., 2003). However, there was no significant difference in RANKL-induced osteoclastogenesis between the WT and *Blnk*$^{-/-}$ cells (data not shown), suggesting that another molecule(s) compensates for the loss of BLNK. SLP-76 (encoded by *Lcp2*) is an adaptor protein homologous to BLNK, functioning mainly in T cells (Pivniouk and Geha, 2000). Since there was no significant difference in RANKL-induced osteoclastogenesis between the WT and *Lcp2*$^{-/-}$ cells (data not shown), we analyzed mice doubly deficient in BLNK and SLP-76 (*Blnk*$^{-/-}$*Lcp2*$^{-/-}$ mice). Although *Blnk*$^{-/-}$ mice frequently develop acute leukemia (Jumaa et al., 2003), there was a normal number of CD11b^{+} osteoclast precursor cells in the M-CSF-stimulated bone marrow cells from *Blnk*$^{-/-}$*Lcp2*$^{-/-}$ mice (Figure 5D), and


A
Sham
OVX
WT
Tec-/- Btk-/-
0.5 mm
Bone volume /tissue volume (%)
Trabecular thickness (μm)
Trabecular separation (μm)
Trabecular number (/mm)
Osteoclast number /bone perimeter (/100 mm)
Eroded surface /bone surface (%)
Sham OVX Sham OVX
WT Tec-/-Btk-/-
B
Control
LPS
0.2 mm
Osteoclast number (/mm)
Eroded surface/bone surface (%)
Increase in marrow cavity (%)
Inflammatory cell layer (mm)
n.s.
C
Control
LPS
LPS +LFM-A13
Increase in marrow cavity (%)
D
RANKL
RANKL+LFM-A13
Serum Ca2+ level (mg/ml)
LFM-A13

the rate of proliferation or apoptosis in $Blnk^{-/-}Lcp2^{-/-}$ BMMs was not significantly different from that in WT BMMs (Figure 5E). We found that in vitro osteoclast differentiation was severely abrogated in $Blnk^{-/-}Lcp2^{-/-}$ BMMs (Figure 5F), suggesting an important role for BLNK and SLP-76 in osteoclast differentiation. Thus, the scaffold proteins, with which the Tec kinases form a complex, have emerged as critical mediators of osteoclastogenic signals. These results further lend support to the crucial role of the RANKL-stimulated formation of the osteoclastogenic complex: the interaction of Tec kinases and their scaffold proteins results in the efficient phosphorylation of PLCγ.

Tec Kinases as Potential Therapeutic Targets for Bone Diseases

To investigate the role of Btk and Tec in the pathological activation of osteoclastogenesis, $Tec^{-/-}Btk^{-/-}$ mice were subjected to an ovariectomy (OVX)-induced model of postmenopausal osteoporosis (Aoki et al., 2006). The bone volume and trabecular bone number/connectivity were significantly reduced by the estrogen withdrawal in WT mice, but such a reduction was observed to a much lesser extent in $Tec^{-/-}Btk^{-/-}$ mice (Figure 6A and Figure S4). An increase in osteoclast number induced by OVX was also much lower in $Tec^{-/-}Btk^{-/-}$ mice (Figure 6A and Figure S4), suggesting a key role of Btk and Tec in the pathological activation of osteoclastogenesis.

Furthermore, $Tec^{-/-}Btk^{-/-}$ mice were subjected to a lipopolysaccharide (LPS)-induced model of inflammatory bone destruction (Takayanagi et al., 2000). $Tec^{-/-}Btk^{-/-}$ mice were protected from the activation of osteoclastogenesis and bone loss (Figure 6B), whereas the formation of an inflammatory cell layer, the number of infiltrated inflammatory cells, IKK activation, and serum levels of TNF-α and IL-6 in $Tec^{-/-}Btk^{-/-}$ mice were not different from those in WT mice (Figure 6B and Figure S5). These results prompted us to examine the therapeutic effects of Tec kinase inhibitor in disease models. Local administration of the Tec kinase inhibitor LFM-A13 had a marked therapeutic effect on the excessive osteoclast formation and bone destruction induced by LPS (Figure 6C) without affecting IKK activation or inflammatory cytokine levels in the serum (Figure S5), although it has been reported that Tec kinases are involved in the activation of immune cells, including lymphocytes and macrophages (Horwood et al., 2003; Mangla et al., 2004). These results suggest that the therapeutic effects of Tec kinase inhibitor in an LPS-induced model are not due to attenuated immune responses but rather are mainly caused by direct inhibitory effects on osteoclast precursor cells. It has been consistently reported that Btk is not essential for LPS-induced inflammatory cytokine production in macrophages under certain conditions (Hata et al., 1998; Perez de Diego et al., 2006).

To determine whether the Tec kinase inhibitor has therapeutic efficacy in a model of osteoporosis, we treated mice that were intraperitoneally injected with GST-RANKL. GST-RANKL injection resulted in an increase in osteoclast number and serum calcium concentration and a decrease in trabecular bone volume, but LFM-A13 treatment significantly ameliorated RANKL-induced bone loss (Figure 6D). These results demonstrate the inactivation of Tec family kinases to be a novel strategy for suppressing osteoclastogenesis in vivo.

DISCUSSION

RANKL-Induced Formation of the Osteoclastogenic Signaling Complex

An osteopetrotic phenotype in $Tec^{-/-}Btk^{-/-}$ mice revealed these two kinases to play a crucial role in the regulation of osteoclast differentiation. Btk and Tec are known to play a key role in proximal BCR signaling (Ellmeier et al., 2000), but this study establishes their crucial role in the integration of the two essential osteoclastogenic signals, RANK and ITAM (Figure 7). Thus, although immune and bone cells share components of signaling cascades, they play distinct roles in each cell type. Furthermore, this study identified an osteoclastogenic signaling complex composed of Tec kinases and adaptor proteins that may provide a new paradigm for the signal transduction mechanism of osteoclast differentiation: ITAM phosphorylation results in the recruitment of Syk, which phosphorylates adaptor proteins such as BLNK and SLP-76, which in turn function as scaffolds to recruit the Tec kinases activated by RANK and PLCγ to the osteoclast signaling complex so as to induce maximal calcium influx.

Such complexes are similar to those formed in the immunological synapse in T cells, which are associated with membrane rafts (Cherukuri et al., 2001). It has been reported that RANK accumulates in membrane rafts, and these specialized domains may play an important role in the RANK signal transduction (Ha et al., 2003). We observed that DAP12, Btk, BLNK, and PLCγ, as well as RANK, were recruited to caveolin-rich membrane domains, which are the crucial signaling domains contained in lipid rafts, after RANKL stimulation (Figure S6). Thus, it is likely that the complex containing both the RANK and ITAM signaling pathways is generated by RANKL stimulation and contributes to the facilitation of the osteoclastogenic signal transduction.

Linkage between Primary Immunodeficiency and Bone Homeostasis

The mutation of *Btk* in humans causes XLA, which is characterized by an arrest in B cell development and immunodeficiency

Figure 6. Tec Kinases as Potential Therapeutic Targets in Bone Diseases

(A) OVX-induced bone loss in WT and $Tec^{-/-}Btk^{-/-}$ mice (evaluated in the femurs and tibiae 3 weeks after sham operation or OVX). The trabecular thickness, separation, and number were obtained from three-dimensional microstructural analysis by microcomputed tomography. Bone volume, osteoclast number, and eroded surface were based on the bone morphometric analysis.

(B) Inflammation-induced bone destruction in WT and $Tec^{-/-}Btk^{-/-}$ mice. Histology of the calvarial bone injected with saline (control) or LPS in WT and $Tec^{-/-}Btk^{-/-}$ mice (TRAP and hematoxylin staining).

(C) Effect of local administration of the Tec kinase inhibitor LFM-A13 (20 mg/kg) on LPS-induced osteoclast formation and bone destruction (TRAP and hematoxylin staining). This inhibitor at this dosage had marginal effects on B cells.

(D) Effect of local administration of LFM-A13 (20 mg/kg) on RANKL-induced osteoclast formation and bone loss. After mice were sacrificed 1.5 hr after the final injection, serum calcium level was measured, and three-dimensional microstructural analysis (femur) and the bone morphometric analysis (tibia) were performed.