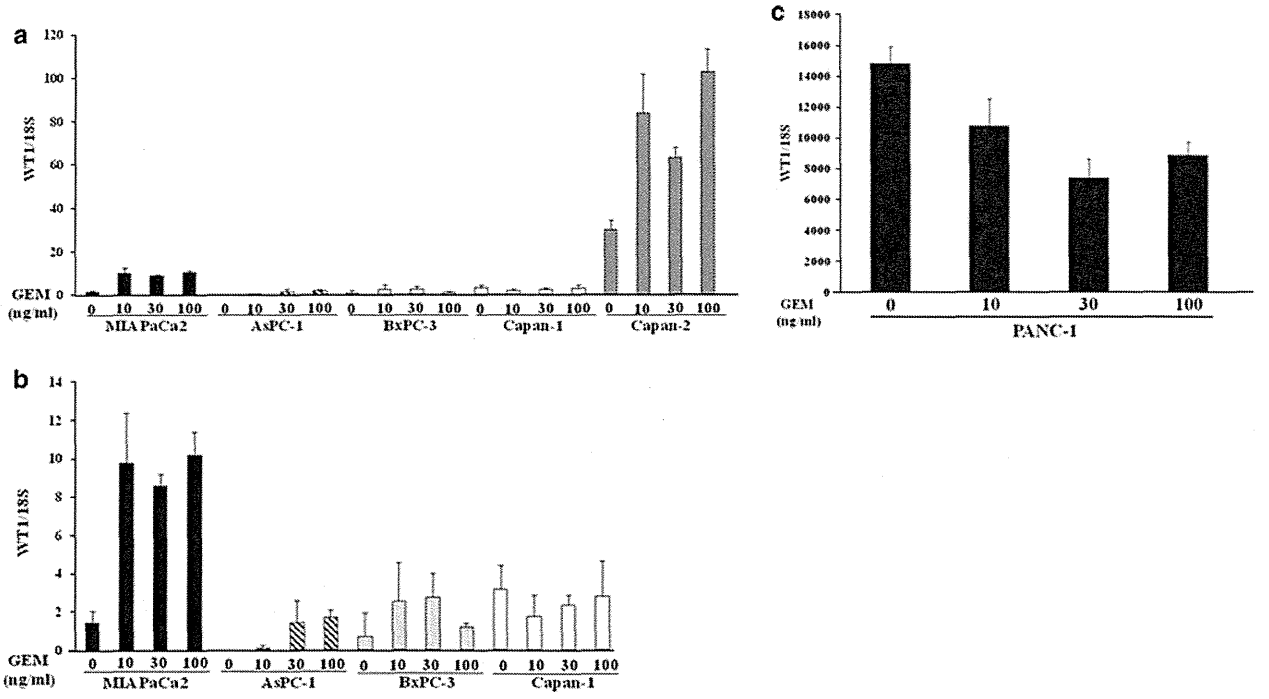

Fig. 2 **a** Up-regulation of WT1 mRNA levels in various human PC cell lines following GEM treatment. Human PC cells (1×10^6 MIAPaCa2, AsPC-1, BxPC-3, Capan-1 or Capan-2) were seeded in 10-cm culture plates. After 24-h incubation, medium was changed to media containing GEM (10, 30 or 100 ng/ml). After 48 h, we used qRT-PCR to quantify the relative ratio of WT1 to 18S mRNA levels in each cell line ($n = 3$). **b** GEM-induced up-regulation of WT1 mRNA in human PC cells with low basal levels of WT1 mRNA (MIAPaCa2, AsPC-1, BxPC-3 and Capan-1). To illustrate these results, we replotted data from (**a**) to represent a considerably narrower range of mRNA level ratios (0–14) on the *y*-axis. (**c**) Expression of WT1 mRNA in human PC cells with high basal levels of WT1 mRNA (PANC-1). To illustrate the results, we plotted data to represent a considerably wider range of mRNA level ratios (0–18,000) on the *y*-axis

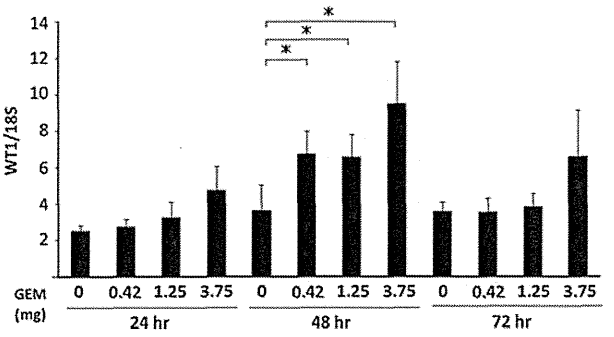

Fig. 3 Tumors in PC-bearing SCID mice treated with GEM show increased WT1 mRNA levels. Ten days after subcutaneous inoculation of SCID mice with 5×10^6 MIAPaCa2 cells (formation of approximately 1-cm diameter tumors), mice were injected intraperitoneally with GEM (0, 0.42, 1.25 and 3.75 mg/mouse). Tumors were resected every 24 h thereafter, and relative levels of WT1 mRNA were quantified using qRT-PCR ($n = 3$). Duplicate trials of the same protocol showed similar results. *$P < 0.01$

(Fig. 5a). Decline in WT1 protein levels following GEM treatment was also observed in immunoblot analyses of the nuclear fraction of treated MIAPaCa2 cells (Fig. 5b).

Enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide following GEM treatment

Figure 6a shows typical standard curve obtained with increasing quantities of WT1 antigenic peptide. The data indicate a linear relation over a wide range (0–1,000 pmol) of analyte amount with correlation coefficients greater than 0.99. The data in the Fig. 6b demonstrate the sensitivity as well as the noise background of the LC–MS/MS. The noise background is less than 1 cps. The signal from injection of 10 pmol of this peptide spiked to MIAPaCa2 cells is approximately 16 cps, giving an S/N ratio of approximately 16. The low noise background and signal of 10 pmol of this peptide indicated the extrapolated limit of detection is less than 0.8 pmol on column under S/N = 2.

The level of the WT1 antigenic peptide was estimated among MHC class I binding peptides from MIAPaCa2 cells treated with either PBS or GEM to 6.49 pmol/10^8cell or 8.78 pmol/10^8cell, respectively. GEM treatment increased the presentation of HLA-A*2402-restricted WT1 antigenic peptide on MIAPaCa2 cells.

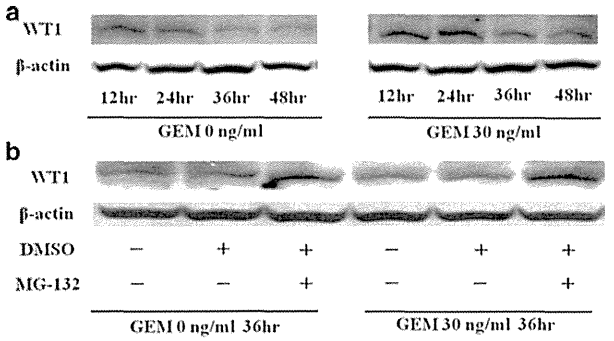

Fig. 4 **a** WT1 protein is degraded by proteasomal enzymes. Twenty-four hours after 3×10^5 MIAPaCa2 cells/well were seeded in 6-well culture plates, medium was exchanged from untreated to media containing GEM (0 or 30 ng/ml). Expression of WT1 protein in the cells was analyzed every 12 h thereafter from immunoblots described in Sect. "Materials and methods". **b** Protease inhibitors block WT1 degradation. Twenty-four hours after incubating MIAPaCa2 cells with GEM (0 or 30 ng/ml), MG-132 in DMSO or DMSO alone was added to each well at a concentration of 5 μM and 0.05%, respectively. Treated and control cells (in 0.05% DMSO alone) were incubated for 12 h before harvesting cells for immunoblot analysis of WT1 and beta-actin proteins

GEM-treated PC cells are killed efficiently by effector cells transduced with genes encoding a WT1-specific T-cell receptor

The susceptibilities of untreated and GEM-treated MIAPaCa2 cells to WT1-specific cytotoxic effector T cells were compared. The cytotoxic effect of WT1-specific effector cells on MIAPaCa2 cells was enhanced significantly when PC cells were treated with either 10 or 30 ng/ml of GEM for 48 h (Fig. 7). Notably, effector cell cytotoxicity was not enhanced by treatment of PC cells with 100 ng/ml of GEM, although this high dose of GEM was more toxic to PC cells than 10 or 30 ng/ml. Up-regulation of MHC class I in MIAPaCa2 cells by GEM treatment that possibly provides the similar results was not observed (data not shown).

Discussion

In the present study, we demonstrate that expression of WT1 mRNA in human PC cells is enhanced by treatment

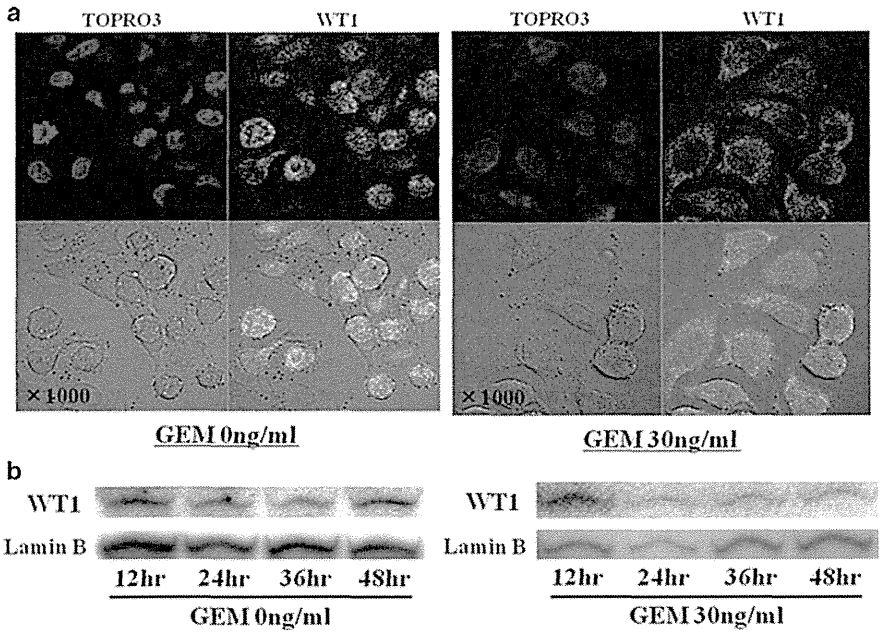

Fig. 5 **a** GEM treatment shifts WT1 protein localization from nucleus to cytoplasm. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, untreated medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). After 24-h incubation, cells were fixed with paraformaldehyde, followed by nuclear staining with TO-PRO-3 iodide (*blue color*) and detection of WT1 with rabbit anti-WT1 polyclonal antibody and anti-rabbit IgG conjugated with fluorescein isothiocyanate (*green color*). Stained cells were observed using confocal microscopy (original magnification ×1,000). **b** GEM treatment diminishes nuclear localization of WT1 protein. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). At 12-hour intervals thereafter, nuclei were isolated and WT1 protein levels of nuclear extracts were analyzed on immunoblots as described in Sect. "Materials and methods"

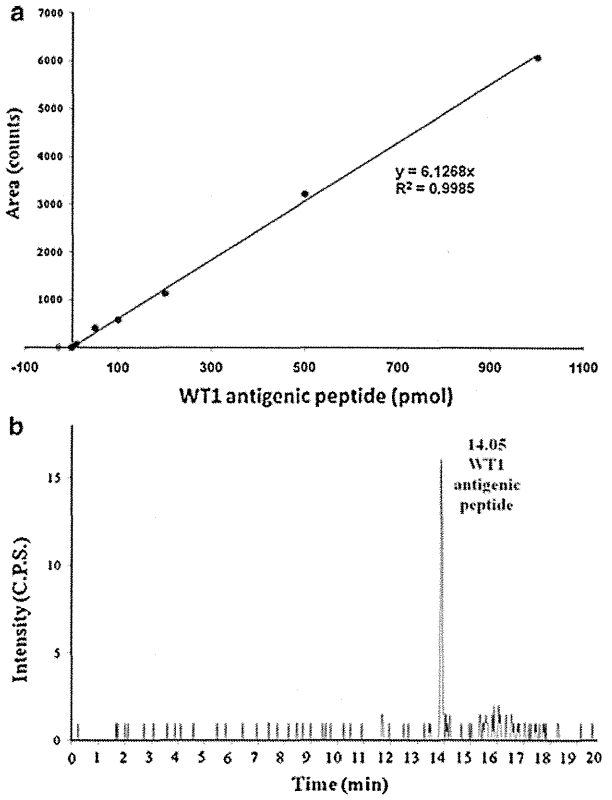

Fig. 6 **a** Standard curve for HLA-A*2402 restricted WT1 antigenic peptide. **b** Trace of MRM signal during LC–MS/MS analysis of spiked HLA-A*2402-restricted WT-1 antigenic standard peptide (10 pmol) in MIAPaCa2 cells

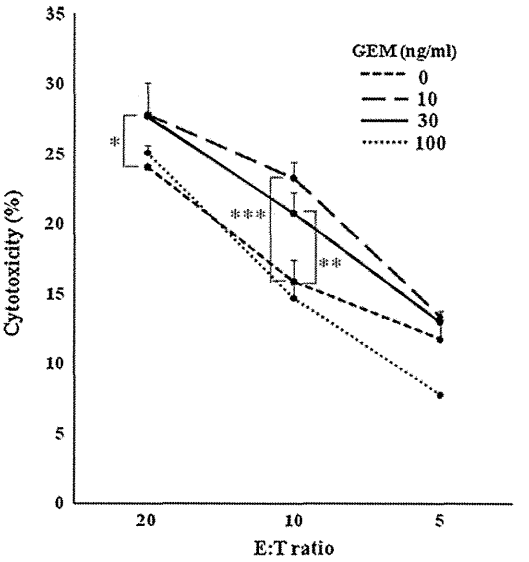

Fig. 7 WT1-specific CTLs kill GEM-treated MIAPaCa2 cells efficiently. MIAPaCa2 cells pretreated with 0, 10, 30, or 100 ng/ml GEM for 48 h were labeled with ^{51}Cr. ^{51}Cr release assays were used to measure the cytotoxic activity of WT1-specific effector cells against untreated or GEM-pretreated MIAPaCa2 cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

with GEM. MIAPaCa2 cells demonstrating GEM-mediated enhancement of WT1 mRNA levels did not proliferate but maintained stable numbers of viable cells with impaired growth by continuous treatment with low-dose GEM as well as short treatment with high-dose GEM. WT1 is a transcription factor with oncogenic potential, in that it can induce malignant cellular phenotypes, suppress apoptosis, and promote cell proliferation [15]. We hypothesize that up-regulation of WT1 levels in PC cells aids cell survival by conferring chemoresistance against GEM's toxic effects.

Based on the fact that GEM-mediated augmentation of WT1 mRNA expression was attenuated by addition of an NF-kB blocking peptide in the culture, activation of NF-kB also appears to play a significant role in WT1 enhancement. NF-kB is known to be active in many malignant tumors and has been implicated in cellular resistance to cytotoxic agents and escape from apoptosis [26]. Previous reports demonstrate that GEM activates NF-kB [27] and that the ensuing regulatory cascade activates the WT1 gene downstream [28]. Human PC cell lines with high NF-kB activity are resistant to GEM [27], and that silencing or suppression of NF-kB increases the sensitivity of PC cells to GEM and induces apoptosis [29–31].

It is of note and interest that some chemotherapeutic agents other than GEM showed capability on up-regulation of WT1mRNA expression. Especially, treatment with oxaliplatin (L-OHP) induced marked enhancement of WT1mRNA expression. Folfirinox including L-OHP was recently reported to be a more efficient regimen for metastatic pancreatic cancer (10). However, combined treatment with Folfirinox and WT1 targeting immunotherapy might be unsuccessful because of severe leukopenia by Folfirinox. GEM has relatively low hematologic toxicity and thus seems to be preferable for combination therapy with WT1 targeting immunotherapy.

We also observed up-regulation of WT1 mRNA by GEM treatment in vivo. Within 48 h of treating MIAPaCa2-bearing SCID mice with a clinical dose of GEM, steady-state levels of WT1 mRNA in the tumor increased. Despite its rapid disappearance after intraperitoneal injection, the enhancement of WT1 mRNA expression in tumor tissue was significant. Enhancement of WT1 mRNA expression was also observed after in vitro short treatment with GEM. These results suggest strongly that GEM treatment of human PC in a clinical setting might induce up-regulation of WT1 in PC cells.

In the present study, we found that the localization of WT1 protein shifted from nucleus to cytoplasm following GEM treatment. WT1 protein has been shown to undergo nucleocytoplasmic shuttling [32], and the function of WT1 has been suggested to correlate with its cellular location: Siberstein et al. [33] described that WT1 was localized to

the cytoplasm and not to nuclei in some human breast cancers and suggested that such localization may be regulated by alternative splicing of WT1 mRNA. On the other hand, immunohistochemical studies of Nakatsuka et al. [34] demonstrate a majority of WT1-positive tumors with diffuse or granular staining in the cytoplasm. Ye et al. [35] report that phosphorylation of WT1 protein resulted in cytoplasmic retention of WT1, thereby inhibiting DNA binding and altering transcriptional activity. Through the activation of NF-kB, GEM treatment may mediate a similar phosphorylation and translocation of WT1 protein from nucleus to cytoplasm.

In order for MHC class I-restricted antigen to be presented and recognized by antigen-specific CTLs, tumor antigen must be degraded by proteasomal enzymes located in the cytoplasm [36]. Retention of an intra-nuclear tumor antigen such as WT1 in the cytoplasm should favor tumor antigen processing, and in fact, we observed enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide using ESI LC–MS/MS analyses. GEM-treated MIAPaCa2 cells showed greater susceptibility than untreated cells to the cytotoxic effects of WT1-specific CTLs generated by transduction of a gene encoding a WT1-specific T-cell receptor. Importantly, treatment with 10–30 ng/ml of GEM enhanced the susceptibility of MIAPaCa2 cells to CTL, but treatment with 100 ng/ml did not. This phenomenon indicates that the enhanced susceptibility of GEM-treated MIAPaCa2 cells to CTLs is not due to GEM toxicity, but to augmented expression of the WT1 target antigen.

GEM is a nucleoside analog with clinical relevance to the treatment of several solid tumors, including PC; nonetheless, its antitumor effect is limited. We observed significant clinical response in a phase I clinical study of combined treatment against advanced PC using a WT1 peptide vaccine and GEM (manuscript in preparation). The presumed actions of GEM up-regulating WT1 expression in vivo and WT1-specific CTLs killing GEM-treated tumor cells efficiently may prove valuable for the treatment of human PC. It has been reported that GEM may suppress the activity of myeloid-derived suppressor cells that inhibit antitumor immunity [37]. In addition, GEM has been shown to increase the number of dendritic cells in blood without affecting T-cell activity in patients with PC [38]. We propose that combining GEM's proven role as an immunopotentiator with its ability to up-regulate target WT1 expression of PC cells will enhance the susceptibility of PC cells to WT1-specific CTLs. Furthermore, PC cells already acquired GEM resistance by the activation of NF-kB might be injured by WT1-specific CTLs. Assessment of the clinical response to combined therapy with WT1 peptide vaccine and GEM is presently underway.

Acknowledgments This work has been supported by Foundation for Promotion of Cancer Research, Mitsui Life Social Welfare Foundation, Grants-in-Aid for Scientific Research (B) and (C) from the Ministry of Education, Cultures, Sports, Science and Technology of Japan, Grantin-Aid of the Japan Medical Association, Takeda Science Foundation, and Pancreas Research Foundation of Japan.

Conflict of interest There are no financial disclosures of any of the authors to declare.

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Prognostic Impact of Number of Resected and Involved Lymph Nodes at Complete Resection on Survival in Non-small Cell Lung Cancer

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Background: Lymph node (LN) status is a major determinant of stage and survival in patients with lung cancer. In the 7th edition of the *TNM Classification of Malignant Tumors*, the number of involved LNs is included in the definition of pN factors in breast, stomach, esophageal, and colorectal cancer, and the pN status significantly correlates with prognosis.

Methods: We retrospectively investigated the prognostic impact of the number of resected LNs (RLNs) and involved LNs in the context of other established clinical prognostic factors, in a series of 928 consecutive patients with non-small cell lung cancer (NSCLC) who underwent complete resection at our institution between 2000 and 2007.

Results: The mean number of RLNs was 15. There was a significant difference in the total number of RLNs categorized between less than 10 and ≥10 ($p = 0.0129$). Although the incidence of LN involvement was statistically associated with poor prognosis, the largest statistically significant increase in overall survival was observed between 0 to 3 and ≥4 involved LNs (hazard ratio = 7.680; 95% confidence interval = 5.051–11.655, $p < 0.0001$). On multivariate analysis, we used the ratio between the number of involved LNs and RLNs. The number of RLNs was found to be a strong independent prognostic factor for NSCLC (hazard ratio = 6.803; 95% confidence interval = 4.137–11.186, $p < 0.0001$).

Conclusion: Complete resection including 10 or more LNs influenced survival at complete NSCLC resection. Four involved LNs seemed to be a benchmark for NSCLC prognosis. The number of involved LNs is a strong independent prognostic factor in NSCLC, and the results of this study may provide new information for determining the *N* category in the next tumor, node, metastasis classification.

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Disclosure: The authors declare no conflicts of interest.

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ISSN: 1556-0864/11/0611-1865

Key Words: Number of resected lymph nodes, Number of involved lymph nodes, Lymph node dissection, Multivariate analysis.

(*J Thorac Oncol.* 2011;6: 1865–1871)

Lung cancer has one of the highest worldwide incidence rates and is the leading cause of cancer-related mortality worldwide.[1] In Japan, lung cancer accounts for 60,000 deaths annually, and surgical resections are performed in approximately 27,000 cases, with an overall survival (OS) rate of 60%, according to the annual reports of the Japanese Association for Thoracic Surgery[2] and the Japanese Lung Cancer Registry.[3]

Various pathological and molecular markers have been assessed regarding their status and role in identifying patients at high risk for recurrence. However, the primary tumor, lymph node (LN), and the metastasis (TNM) staging system remain the most important determinant of outcome. Because the prognosis of lung cancer is directly proportional to the presence of LN metastasis, accurate LN assessment is crucial in determining treatment. The role of hilar and mediastinal lymphadenectomy in the staging and treatment of non-small cell lung cancer (NSCLC) remains controversial. Accurate staging of NSCLC requires assessment of the hilar and mediastinal LNs based on pathologic evaluation. In almost all surgical cooperative group trials and clinical settings in Japan, systematic LN dissection in ipsilateral hilar and mediastinal stations is standard. However, there is continual debate regarding the degree to which hilar and mediastinal LNs should be located and removed.

The number of resected LNs (RLNs) has been proven to have prognostic value in colorectal, breast, and bladder cancer.[4–6] Moreover, the number of involved LNs at the time of surgery currently influences staging. However, these items have not yet been incorporated into the latest 7th edition of the TNM classification of lung cancer.[7]

Therefore, we retrospectively investigated the prognostic impact of the number of RLNs and involved LNs in the context of other established clinical prognostic factors, in a series of 928 consecutive patients with NSCLC who underwent complete

resection at Tokyo Medical University. Specifically, we attempted to clarify the number of LNs that should be resected, and the number of involved LNs needed to make an accurate prognosis.

PATIENTS AND METHODS

Patient Selection

From January 2000 to November 2007, a total of 1311 patients underwent resection for primary lung cancer at our institution. Cases of induction therapy, incomplete resection, and limited resection were excluded from this study. Patients whose tumors were classified histologically as small cell lung cancer or low-grade malignant tumors were also excluded. We retrospectively analyzed the remaining 928 consecutive patients with NSCLC who underwent complete resection with curative intent (minimum procedure of lobectomy) with systematic LN dissection of the hilum and mediastinum according to current surgical methods.[8] Patient charts, including pathologic diagnosis and operative reports, were reviewed. Staging was determined according to the international TNM staging system.[9] The histological tumor type was determined according to the World Health Organization classification, 3rd edition. All dissected LNs were examined pathologically and classified according to anatomical location by the numbering system described in the Naruke map.[10] The number of RLNs and involved LNs was confirmed based on the pathological report provided by M.N., J.M., and T.N. These pathologists were blinded to the clinical outcome.

Patient Characteristics

The characteristics of the 928 consecutive patients who underwent surgery for NSCLC were as follows: age, median (range): 65.0 years (22–87 years); sex: 547 (59.0%) men and 381 (41.0%) women; clinical stages: 768 (82.8%) stage I, 84 (9.1%) stage II, and 76 (8.1%) stage III; pathological stage: 677 (72.9%) stage I, 121 (13.0%) stage II, 129 (13.9%) stage III, and 1 (0.2%) stage IV; histopathological diagnosis: 684 (73.7%) adenocarcinomas, 182 (19.6%) squamous cell carcinomas, 52 (5.6%) large cell carcinomas, and 10 (1.1%) others; surgical procedure: 870 (93.8%) lobectomies, 42 (4.5%) bilobectomies, and 16 (1.7) pneumonectomies. The mean number of RLNs was 15 (right side, 15.5; left side, 14.3); the mean number of involved LNs was 4.2 (0–22) (Table 1). The median follow-up time was 3.5 years.

TABLE 1. Patient Characteristics

Variable	Category	*n* (%)
Age (yr)	Mean	65.0
	Range	22–87
Sex	Men	548 (59.0)
	Women	380 (41.0)
Histopathology	Adenocarcinoma	684 (73.7)
	Squamous cell	182 (19.6)
	Large cell	52 (5.6)
	Other	10 (1.1)
Clinical stage	I	768 (82.8)
	II	84 (9.1)
	III	76 (8.1)
Pathological stage	I	677 (72.9)
	II	121 (13.0)
	III	130 (14.1)
Tumor location	Right side	602 (64.9)
	Upper/middle/lower	334/64/204
	Left side	326 (35.1)
	Upper/lower	190/136
Surgical procedure	Lobectomy	870 (93.8)
	Bilobectomy	42 (4.5)
	Pneumonectomy	16 (1.7)
Total number of resected LNs	Mean (range)	15.0 (1–49)
	0–4	59 (6.4)
	5–9	177 (19.1)
	10–14	251 (27.0)
	15–19	201 (21.6)
	≥20	241 (25.9)
Total number of involved LNs in positive cases	Mean (range)	4.2 (1–22)
	0	724 (78.0)
	1–3	122 (13.1)
	≥4	82 (8.9)

LNs, lymph nodes.

Statistical Analysis

We investigated the association between the total number of RLNs or involved LNs and OS. OS was calculated from the date of surgery to the time of death. Observations were censored at final follow-up if the patient was alive. All patients in this series were categorized into four groups according to the number of RLNs less than 5 versus 5 or more, less than 10 versus 10 or more, less than 15 versus 15 or more, and less than 20 versus 20 or more. On analysis of survival differences based on the number of involved LNs, patients were categorized into groups of those with 0 versus 1 or more, less than 3 versus 3 or more, less than 4 versus 4 or more, and less than 5 versus 5 or more of involved LNs. Survival curves were plotted using the Kaplan-Meier method. Differences in survival among the groups were examined using the log-rank test. A two-category comparison was performed using the Pearson χ^2 test and the Student t test for quantitative data. Multivariate analysis was performed using the Cox proportional hazards model to examine any possible association between the ratio of the total number of RLNs and involved LNs and survival, with adjustment for the effects of other potential prognostic factors, including age, sex, histology, tumor factor, and type of surgery performed. All tests were two sided, and p values of less than 0.05 were considered to represent statistically significant differences. StatView version 5.0 software (SAS Institute Inc., Cary, NC) was used for statistical analysis.

Ethical Considerations

The approval of the Institutional Review Board of Tokyo Medical University was obtained, but as this was a retrospective study the need to obtain written informed consent from either the patients or their representatives was waived, in accordance with the AMA Manual of Style (10th edition).

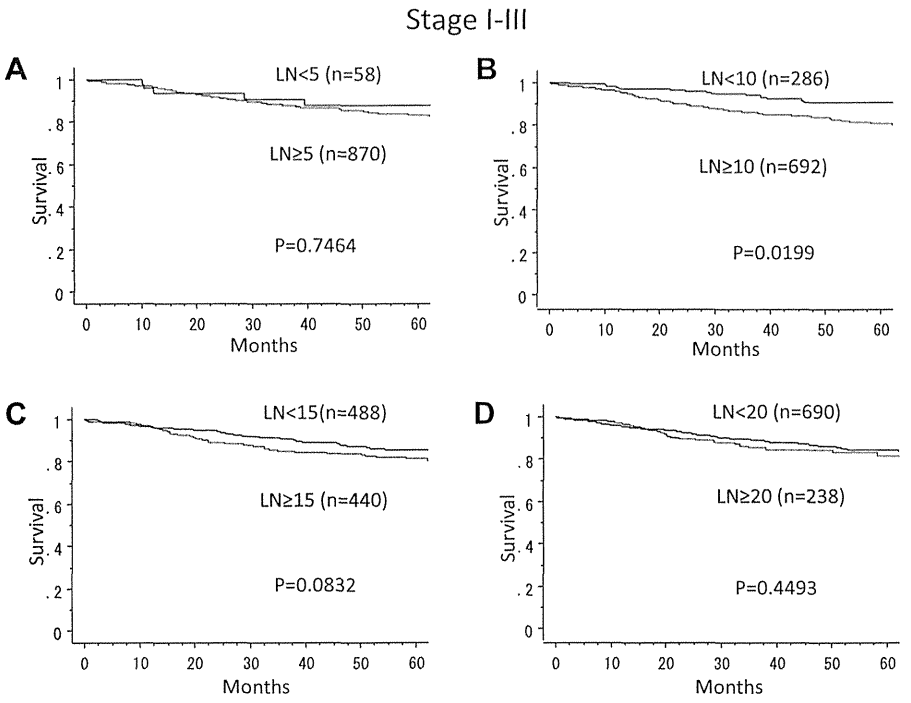

FIGURE 1. Survival curves according to the number of resected LNs at the time of complete resection in patients with stages I to III non-small cell lung cancer. *A*, LN, five nodes as cutoff. *B*, LN, 10 nodes as cutoff; a significant difference in survival was observed. *C*, LN, 15 nodes as cutoff. *D*, LN, 20 nodes as cutoff.

TABLE 2. Correlations between Overall Survival and Total Number of Resected Lymph Nodes

Valuables	*p*	HR	95% CI
5 vs. ≥5	0.7464	1.135	0.528–2.440
6 vs. ≥6	0.5464	1.233	0.624–2.437
7 vs. ≥7	0.1611	1.591	0.831–3.047
8 vs. ≥8	0.744	1.725	0.948–3.140
9 vs. ≥9	0.0217	1.783	1.088–2.923
10 vs. ≥10	0.0199	1.795	1.098–2.912
11 vs. ≥11	0.0295	1.651	1.051–2.595
12 vs. ≥12	0.0473	1.521	1.005–2.302
13 vs. ≥13	0.0907	1.394	0.949–2.050
14 vs. ≥14	0.1137	1.354	0.930–1.973
15 vs. ≥15	0.0832	1.388	0.956–2.014

HR, hazard ratio; CI, confidence interval.

RESULTS

Survival and Number of RLNs

We investigated the prognostic impact of the number of RLNs (mean number of RLNs = 15). Patients were categorized into four representative groups according to the total number of RLNs: less than 5 versus 5 or more, less than 10 versus 10 or more, less than 15 versus 15 or more, and less than 20 versus 20 or more (Figure 1). Table 2 presents each *p* value, hazard ratio (HR), and 95% CI comparing each subgroup categorized according to total number of RLNs. The largest significant difference was found in the total number of RLNs categorized between less than 10 and 10 or more ($p = 0.0199$, HR = 1.795, 95% CI = 1.098–2.912). However, even 15 or more RLNs had no significant prognostic impact on the survival of patients with NSCLC in the present series. There was no sign of incremental improvement in or impairment of survival after the resection and evaluation of 15 or more LNs for curative resection of NSCLC. There were no statistically significant differences in survival according to the total number of RLNs in cases of stage I NSCLC (Figure 2).

As shown in Table 3, the mean numbers of RLNs on both the right and left sides were significantly higher in pN1 or pN2–3 cases than in pN0 cases (right side: $p = 0.0007$, $p = 0.0002$, left side: $p = 0.0068$, $p = 0.0162$, respectively). The mean number of RLNs in cases with right-sided tumors was significantly higher than that in cases with left-sided tumors.

Survival and Number of Involved LNs

We analyzed the number of involved LNs that could provide the most appropriate indicator of OS in NSCLC. Although the incidence of LN involvement was associated with poor prognosis, the largest statistically significant increase in OS was observed between zero to three and four or more involved LNs (HR, 7.680; 95% CI, 5.051–11.655, $p < 0.0001$) (Figure 3). Although patients with no involved LNs had a better outcome than those with 1 to 3 involved LNs, there was no significant difference in survival between the two groups ($p = 0.1831$). Patients with four or more involved LNs had a significantly worse outcome than those with one to three involved LNs ($p < 0.0001$). These results suggest that four or more involved LNs would be the best benchmark of OS in NSCLC (Figure 4).

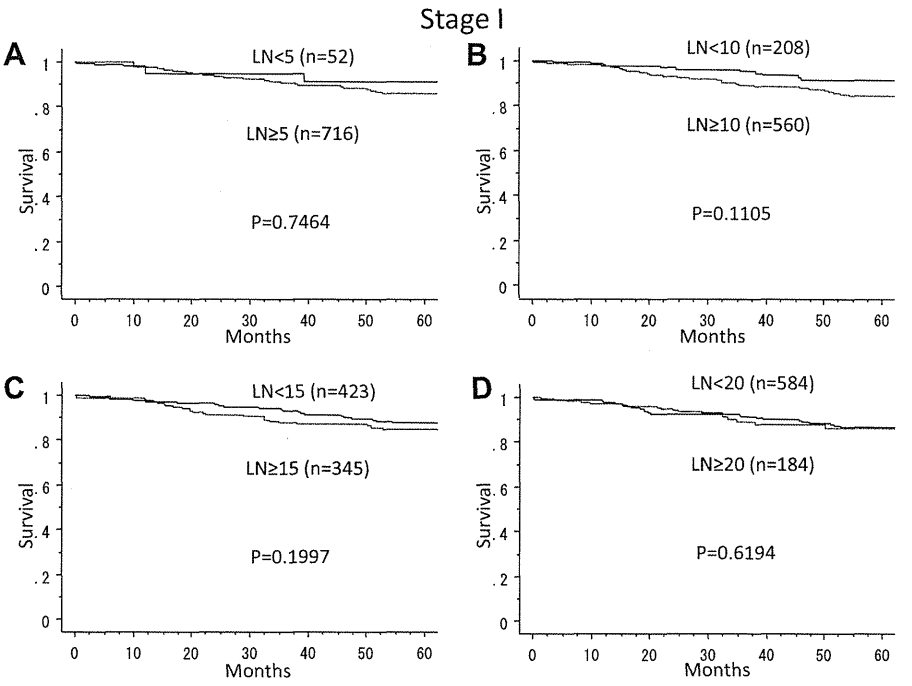

FIGURE 2. Survival curves according to the number of resected LNs at the time of complete resection in patients with stage I, non-small cell lung cancer. *A*, LN, five nodes as cutoff. *B*, LN, 10 nodes as cutoff. *C*, LN, 15 nodes as cutoff. *D*, LN, 20 nodes as cutoff. No significant difference in survival was observed in any group.

TABLE 3. Mean Number of Resected Lymph Nodes on Right or Left Side

	Mean Number	*p*
Right side lymph nodes, $n = 602$		
Total	15.5	
pN0	14.7	
pN1	18.2	0 vs. 1: $p = 0.0007$[a]
pN2–3	19.0	0 vs. 2–3: $p = 0.0002$[a]; 1 vs. 2–3: $p = 0.7199$
Left side lymph nodes, $n = 326$		
Total	14.3	
pN0	13.5	
pN1	16.6	0 vs. 1: $p = 0.0068$[a]
pN2–3	16.3	0 vs. 2–3: $p = 0.0162$; 1 vs. 2–3: $p = 0.8985$
Right vs. left		$p = 0.0323$[a]

[a] Statistical significance.

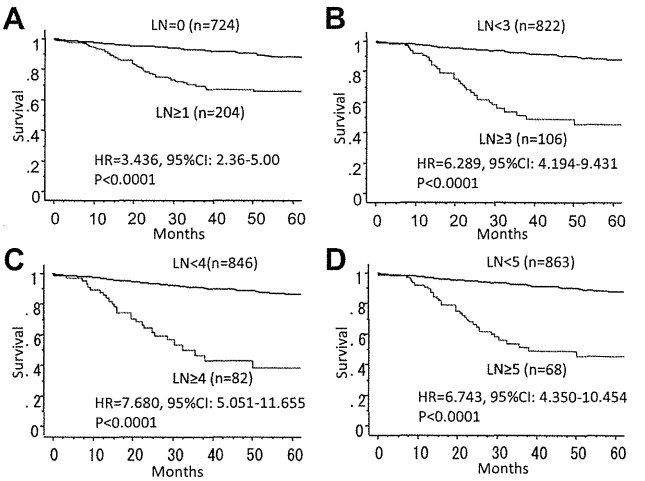

FIGURE 3. Survival curves according to the number of involved LNs at the time of complete resection in patients with stages I to III, non-small cell lung cancer. *A*, LN, one node as cutoff. *B*, LN, three nodes as cutoff. *C*, LN, four nodes as cutoff. *D*, LN, five nodes as cutoff. Although the incidence of lymph node involvement was statistically associated with poor prognosis, the largest statistically significant increase in OS was seen between zero to three and 4 or more involved LNs.

Correlations between Number of RLNs, Involved LNs and pN Status

Before analyzing the possibility of RLNs and involved LNs as possible independent prognostic factors by multivariate analysis, we examined whether RLNs, involved LNs and pN status were confounding factors. The mean and range of the total number of RLNs in our series were 15.0 and 1 to 49, respectively. The mean number of RLNs was significantly increased in pN1 or pN2–3 cases compared with pN0 cases ($p < 0.0001$ and $p < 0.0001$, respectively), whereas the mean and range of the total number of involved LNs in our pN-positive series were 4.2 and 1 to 22, respectively. The mean numbers of involved LNs in pN1 and pN2–3 cases were 2.15 and 6.56, respectively. The number of involved LNs was significantly higher in pN2–3 cases than in pN1 cases ($p < 0.0001$). These results demonstrate that each of these prognostic factors (i.e., the number of RLNs and involved LNs,

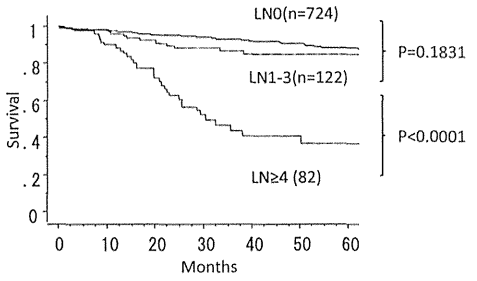

FIGURE 4. Survival curves according to the number of involved LNs at the time of complete resection in patients with stages I to III, non-small cell lung cancer. Although patients with no involved LNs had a better prognosis than those with one to three involved LNs, there was no significant difference in survival between the two groups. Patients with four or more involved LNs had a significantly worse outcome than those with one to three involved LNs.

and pN status) were confounding factors in our series. Therefore, in the subsequent multivariate survival analysis, we used the ratio between the number of involved and RLNs to reflect both factors in the multivariate analysis similarly to a previously reported method.[11]

Multivariate Survival Analysis

We performed multivariate analysis to confirm the prognostic impact of the total number of RLNs and involved LNs in NSCLC, using the ratio between the number of involved and RLNs, according to a previously observed correlation.[11] As shown in Table 4, RLNs strongly correlated with poor prognosis on multivariate analysis after adjustments for sex, age, histology, tumor factor, and surgical procedure. We therefore concluded that RLN was a strong independent prognostic factor for NSCLC (HR, 6.803; 95% CI, 4.137–11.186, $p < 0.0001$). Other independent prognostic factors identified on multivariate analysis included sex (HR, 0.620; 95% CI 0.401–0.958, $p = 0.0313$), age (HR, 1.598, 95% CI 1.090–2.341, $p = 0.0162$), and T factor (HR, 0.392, 95% CI, 0.256–0.600, $p < 0.0001$).

DISCUSSION

We set out to determine the number of LNs that should be resected, and the number of involved LNs for the accurate prediction of outcome in resectable cases of lung cancer. Opinions still vary among surgeons as to whether to remove all, some, or none of the mediastinal LNs at the time of pulmonary resection for lung cancer, and practices vary worldwide. In almost all surgical cooperative group trials in North America, LN sampling is standard, whereas systematic LN dissection is standard in Japan.

LN status is a major determinant of stage and survival in patients with lung cancer. However, the role of mediastinal lymphadenectomy in the staging and treatment of NSCLC remains controversial. The present results indicate that patient survival after complete NSCLC resection is associated with the number of LNs harvested during surgery. The largest significant difference was observed in the total number of RLNs categorized between less than 10 and 10 or more ($p = 0.0199$, HR = 1.795, 95% CI = 1.098–2.912). Patients with 10 or more RLNs had significantly worse outcomes than those with less than 10 RLNs (Figure 1), contrary to the findings of previous studies of stage I NSCLC cases.[11–13] As shown in Table 3, the mean number of RLNs on both the right and left sides was significantly higher in pN1 or pN2–3 cases than in pN0 cases (right side: $p = 0.0007$, $p = 0.0002$; left side: $p = 0.0068$, $p = 0.0162$, respectively), which may be one reason why patients with NSCLC with 10 or more RLNs had a worse outcome than those with less than 10 RLNs. According to the results of the American College of Surgeons Oncology Group (ACOSOG) Z0030 study, a higher N stage was also associated with increased LN removal (N0: 19.2 ± 10.1; N1: 22.8 ± 10.9; N2: 24.5 ± 10.8; $p = 0.043$).[12] This is possibly because surgeons tend to harvest more LNs in patients with LN-positive disease at the time of surgery, in expectation of therapeutic benefit. However, even 15 or more

TABLE 4. Univariate and Multivariate Survival Analyses

Variable	Category	*n*	Univariate Analysis *p*	Multivariate Analysis HR	95% CI	*p*
Sex	Men	548	0.011[a]	0.620	0.401–0.958	0.0313[a]
	Women	381				
Age (yr)	<70	690	0.0209[a]	1.598	1.090–2.341	0.0162[a]
	≥70	338				
Histopathology	Non-adenocarcinoma	244	0.015[a]	0.790	0.518–1.203	0.2719
	Adenocarcinoma	684				
T factor	T2–3	433	<0.0001[a]	0.392	0.256–0.600	<0.0001[a]
	T1	495				
Surgical procedure	Lobectomy	912	0.0136[a]	2.521	0.768–8.280	0.1273
	Pneumonectomy	16				
RLNs	<0.4	882	<0.0001[a]	6.803	4.137–11.186	<0.0001[a]
	≥0.4	46				

[a] Statistical significance.
RLNs, ratio between the number of involved and resected lymph nodes; CI, confidence interval; HR, hazard ratio.

RLNs had no significant impact on OS in patients with NSCLC in the present series, contrary to the results of a previous large study.[13] There appeared to be neither incremental improvement nor impairment of survival after resecting and evaluating 15 or more LNs with curative intent in NSCLC in the current series. One possible explanation for this is that the presence of approximately 10 dissected LNs increases the staging accuracy.

There was no significant difference in survival according to the total number of RLNs in stage I NSCLC in the current series. Recent retrospective studies from cancer registries,[14] nonrandomized trials,[15] and other institutions,[16–21] have indicated that the number of RLNs is associated with better OS.[16–19] Although LN removal may be therapeutic, the therapeutic benefit is likely to be small for patients with stage IA NSCLC, because all LNs in stage IA should be negative. The other, less likely explanation, is that a more extensive LN dissection such as systematic mediastinal LN dissection may be therapeutic, at least in stage I NCSLC.

The present analysis shows that an increasing number of RLNs during complete NSCLC resection is associated with a statistically significant difference in survival, which peaks at 10 to 14 LNs. Some studies have recommended that the minimum requirements for accurate nodal staging must include the removal of at least six LNs from hilar and mediastinal stations.[7,22,23] However, others have recommended the examination of a minimum of 10 LNs and at least three LN stations.[14,19] Although we are reluctant to recommend a definitive optimal number of LNs, the current data support the conclusion that an evaluation of nodal status should include at least 10 LNs.

Nodal involvement is the most important prognostic factor in determining survival for many malignant tumors. These factors are represented by the N category in the TNM classification and are grouped according to the anatomical location and/or number of LN involvement. In the most recently published 7th edition of the *TNM Classification of Malignant Tumors* (2009),[24] the number of involved LNs is included in the definition of pN factors in breast, stomach, esophageal, and colorectal cancer, and pN status shows a significant correlation with outcome. The nodal system in this edition in lung cancer is still based on the anatomical location of involved LNs. The Naruke map and the American Thoracic Society map have been combined into the International Association for the Study of Lung Cancer map, and the definition of the border between N1 and N2 has been changed, because of its complexity and ambiguity. However, this change is based on the anatomical location, not on the biological issue. In the current study, we predicted patient outcome after complete NSCLC resection according to the number of involved and RLNs, as previous reports have suggested.[25,26] Recently, Asamura and coworkers[27] have provocatively suggested that the number of metastatic LNs provides more accurate pathologic nodal staging than the current method of considering anatomical location of involved nodes. The largest statistically significant increase in OS was observed between zero to three and four or more involved LNs (HR, 7.680; 95% CI, 5.051–11.655; $p < 0.0001$) (Figure 2). Therefore, the current data indicate that four or more involved LNs serve as a good indicator of outcome after complete NSCLC resection. Because it is possible that the number of RLNs and involved LNs may indicate the quality of surgery in the determination of accurate staging and survival impact after complete NSCLC resection, we used RLNs as a prognostic predictor on multivariate analysis. In addition to T stage, RLNs had a strong independent effect on survival in patients with complete NSCLC resection in the present study. Indeed, the 5-year survival ratio of patients with RLNs ≥ 4 is similar to that of patients with pN2 disease in our series (data not shown). Although the nodal classification according to the number of involved LNs is simple and easy to be incorporated in the next TNM classification, there are a few limitations that are not helpful in deciding treatment preoperatively because it is mainly based on pathological assessment. However, this may change in the future with the development of new imaging device.

Our data suggested that the number of involved LNs expands pN category information and may provide additional information for the pN category of the next TNM classification. Further large-scale cohort studies, including global prospective validation analyses and multi-institutional studies are warranted.

CONCLUSION

We retrospectively evaluated the prognostic impact of the number of RLNs and involved LNs on the survival of patients with complete NSCLC resection. We found that 10 or more LNs harvested with complete LN dissection possibly influenced survival after complete NSCLC resection. Moreover, the presence of four involved LNs seemed to be a good indicator of outcome after complete NSCLC resection. The number of involved LNs was a strong independent prognostic factor in NSLC, and this may provide new information for the N categorization of the next TNM classification.

ACKNOWLEDGMENTS

Supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (21791332) and the Ministry of Health, Labour and Welfare (22101601).

The authors thank Mr. Roderick J. Turner, Assistant Professor Edward F. Barroga and Professor J. Patrick Barron, Chairman of the Department of International Medical Communications at Tokyo Medical University for their review of the English manuscript.

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bjh research paper

CD48 as a novel molecular target for antibody therapy in multiple myeloma

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Received 9 August 2011; accepted 11 October 2011

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Summary

Monoclonal antibody (mAb) drugs are desirable for the improvement of multiple myeloma (MM) treatment. In this study, we found for the first time that CD48 was highly expressed on MM plasma cells. In 22 out of 24 MM patients, CD48 was expressed on more than 90% of MM plasma cells at significantly higher levels than it was on normal lymphocytes and monocytes. CD48 was only weakly expressed on some $CD34^+$ haematopoietic stem/progenitor cells, and not expressed on erythrocytes or platelets. We next examined whether CD48 could serve as a target antigen for mAb therapy against MM. A newly generated in-house anti-CD48 mAb induced mild antibody-dependent cell-mediated cytotoxicity and marked complement-dependent cytotoxicity against not only MM cell lines but also primary MM plasma cells *in vitro*. Administration of the anti-CD48 mAb significantly inhibited tumour growth in severe combined immunodeficient mice inoculated subcutaneously with MM cells. Furthermore, anti-CD48 mAb treatment inhibited growth of MM cells transplanted directly into murine bone marrow. Finally and importantly, we demonstrated that the anti-CD48 mAb did not damage normal $CD34^+$ haematopoietic stem/progenitor cells. These results suggest that the anti-CD48 mAb has the potential to become an effective therapeutic mAb against MM.

Keywords: multiple myeloma, CD48, monoclonal antibody, antibody therapy, xenograft model.

The past decade has seen major advances in the treatment of multiple myeloma (MM) (Kyle & Rajkumar, 2004; Raab *et al*, 2009). Thalidomide (Singhal *et al*, 1999), bortezomib (Richardson *et al*, 2003) and lenalidomide (Dimopoulos *et al*, 2007) have all emerged as very effective drugs for MM. In addition, autologous haematopoietic stem cell transplantation

First published online 21 November 2011
doi:10.1111/j.1365-2141.2011.08941.x

has improved complete response rates and has prolonged median overall survival for MM patients (Attal *et al*, 1996). However, MM is still not a curable disease, and that novel therapeutic drugs continue to be needed to cure MM patients (Hideshima *et al*, 2007).

Monoclonal antibody (mAb) therapy has had a major effect on the therapy of haematological malignancies. An0074i-CD20 mAb (rituximab) has emerged as a therapeutic agent and has resulted in improved prognosis for B cell lymphoma (Maloney *et al*, 1994). Given that the mechanisms of cytotoxicity by mAb therapy are quite different from those of chemotherapeutic drugs, mAb therapy can work synergistically with chemotherapy. In fact, administration of rituximab in combination with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisolone) for B cell lymphoma patients resulted in major improvement in patient prognosis (Coiffier *et al*, 2002). These findings suggest the importance of developing therapeutic mAbs against MM to improve the prognosis of MM patients. However, as yet there are no approved immunotherapeutic options for MM. A number of therapeutic mAbs against MM, such as anti-CD40 (Tai *et al*, 2005), CD38 (Stevenson *et al*, 1991; Ellis *et al*, 1995; van der Veer *et al*, 2010; de Weers *et al*, 2011), CD138 (Tassone *et al*, 2004a), CD56 (Tassone *et al*, 2004b), β2 microglobulin (Yang *et al*, 2006), FGFR3 (Hadari & Schlessinger, 2009) and CS-1 mAb (Hsi *et al*, 2008; Tai *et al*, 2008), have demonstrated significant anti-tumour activity in preclinical models *in vivo*, and some of them are now being tested in clinical trials (Ocio *et al*, 2008).

Ideally, targets for therapeutic mAbs should be specifically expressed on malignant cells but not on normal cells. In reality, the paucity of tumour-specific antigens has led to the development of mAbs directed against cell surface molecules characteristic of the lineage from which the malignant cells derive, as in the case with mAbs against CD20 (rituximab) (Maloney *et al*, 1994) for B cell lymphoma and CD52 (alemtuzumab) for chronic lymphocytic leukaemia (CLL) (Keating *et al*, 2002). As these mAbs are not specific for malignant cells and deplete normal haematopoietic cells together with malignant cells, haematological toxicity is inevitable. In particular, treatment with alemtuzumab, the mAb against CD52, which is broadly expressed by mature leucocytes, has reportedly led to severe immune-deficiency (Ghobrial *et al*, 2003; Kluin-Nelemans *et al*, 2008). However, alemtuzumab can still be tolerated if accompanied by appropriate prophylaxis for virus infection (O'Brien *et al*, 2008) and thus can benefit CLL patients (Moreton *et al*, 2005; Hillmen *et al*, 2007), because normal haematopoietic stem cells (HSCs) and haematopoietic progenitor cells (HPCs), which are negative for CD52, can reestablish a normal haematopoietic system after discontinuation of the mAb treatment. These findings suggest that cell surface molecules expressed on plasma cells but not on HSCs and HPCs constitute potential candidates as targets for therapeutic mAbs against MM.

CD48 is a 47-kD glycophosphatidylinositol-linked glycoprotein that is expressed on mature lymphocytes and monocytes, but not on non-haematopoietic tissues (Vaughan *et al*, 1983). Because it was known that CD48 is expressed in lymphoid leukaemia and lymphoma, the potentials of anti-CD48 mAb as a therapeutic tool against these diseases were previously tested. Murine IgM anti-CD48 mAb was used in the treatment of patients with B cell-chronic lymphocytic leukaemia, and transient clinical responses were observed (Greenaway *et al*, 1994). Murine IgG2a and chimeric mAb against CD48 showed significant anti-tumour activity against Raji B cell leukaemia cell line (Sun *et al*, 1998, 2000). In the present study, we shed light on CD48 as a novel target for therapeutic mAb against MM. CD48 expression on MM or normal plasma cells had not been reported previously. We demonstrated that the cell surface protein CD48 is constitutively expressed on almost all MM plasma cells at significantly higher levels than on $CD34^+$ HSCs/HPCs and normal leucocytes. Furthermore, we generated a novel anti-CD48 mAb that could induce cytotoxicity against MM cells *in vitro*. The *in vivo* anti-MM potency of the anti-CD48 mAb was tested in mouse xenograft models. We also examined whether the anti-CD48 mAb damages normal HSCs and HPCs.

Methods

Patient samples

Bone marrow (BM) cells from MM patients were collected from iliac bone after informed consent had been obtained. Mononuclear cells were purified using Ficoll Paque (GE Healthcare, Piscataway, NJ, USA), and subjected to analyses. The research was approved by the institutional review boards of Osaka University School of Medicine and of all the hospitals participating in this study.

Cell lines

OPM2 cells (Katagiri *et al*, 1985) were kindly gifted from Yuzuru Kanakura (Osaka University, Japan). MM1S cells were kindly gifted from Hiroshi Yasui (Sapporo Medical College, Japan). RPMI8226 cells and U266 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Flow cytometry and cell sorting

Single cell suspensions from BM were stained with CD48-fluorescein isothicyanate (FITC) (MEM-102; Biolegend, San Jose, CA, USA or ebio156-4H9; eBioscience, San Jose, CA, USA) or CD52-FITC (eBioscience), and other fluorochrome-conjugated mAbs. Analysis and cell sorting were performed on FACS Aria (BD Biosciences, San Jose, CA, USA). Annexin V-allophycocyanin (APC) (Biolegend) was used to identify apoptotic cells.

Generation of anti-CD48 mAbs

BaF3 cells expressing human CD48 (hCD48) cDNA (Toyobo, Tokyo, Japan) were generated by retrovirus transduction. Six-week-old Balb/c mice (CLEA Japan, Tokyo, Japan) were immunized by footpad injection of hCD48-expressing BaF3 cells. Lymphocytes from popliteal lymph nodes were fused with SP2/0 mouse myeloma cells by using polyethylene glycol (Roche Applied Science, Basel, Switzerland). To identify anti-hCD48 mAbs secreting hybridoma clones, NIH3T3 cells expressing hCD48-ires-GFP were stained first with hybridoma supernatants, then with phycoerythrin (PE)-conjugated anti-mouse IgG antibody (eBioscience), and analysed by fluorescence-activated cell sorting (FACS).

In vitro cytotoxicity assay

Antibody-dependent cell-mediated cytotoxicity (ADCC) was measured by a ^{51}Cr release assay. Target cells labelled with ^{51}Cr were incubated with either anti-CD48 mAb (1B4) or isotype control (10 μg/ml) at 37°C for 15 min. Splenocytes from severe combined immunodeficient (SCID) mice (CLEA Japan, Inc., Tokyo, Japan) were then added as effector cells, and incubated at 37°C for 4 h. Finally, the ^{51}Cr released in the supernatants was counted with a gamma counter. Complement-dependent cytotoxicity (CDC) was also measured with a ^{51}Cr-release assay. The aliquots of the labelled cells were incubated with either anti-CD48 mAb (1B4) or isotype control (10 μg/ml) for 15 min. Cells were then incubated in RPMI 1640 medium supplemented with 25% baby rabbit complement (Cedarene, Burlington, Canada) for 1·5 h at 37°C. The percentage of specific lysis was calculated according to the following formula: percentage of specific lysis = $(E - S)/(M - S) \times 100$, where E is the experimental release, S is the spontaneous release, and M is the maximum release by 1% Triton X-100. Purification of CD138$^+$ plasma cells from BM cells from MM patients or CD8$^+$ T cells from peripheral blood of healthy individuals was performed using CD138 micro beads (Miltenyi Biotec, Gladbach, Germany) or IMag human CD8 T lymphocyte enrichment set (BD Bioscience), according to the manufacturers' instructions.

In vivo xenograft mouse models

Six-to-eight-week-old female SCID mice (CLEA Japan, Inc.), Rag2$^{-/-}\gamma c^{-/-}$ mice (Goldman *et al*, 1998) or non-obese diabetic (NOD)/SCID mice (CLEA Japan, Inc.) were subcutaneously inoculated with 5×10^6 OPM2 cells into the lower left flank. Tumour volume was calculated by means of caliper measurements using the following formula: $L \times W \times H/2$, where L (length) is the longest side of the tumour in the plane of the animal's flank, W (width) is the longest measurement perpendicular to the length and in the same plane, and H (height) is taken at the highest point perpendicular to the flank of the animal. Intra-BM transplantation was performed as previously reported (Wang *et al*, 2003) with OPM2 MM cells. Eight-week-old female Rag2$^{-/-}\gamma c^{-/-}$ mice irradiated with 200cGy 4–24 h before transplantation were injected with OPM2 MM cells into the left tibia.

Colony forming assay

Methylcellulose culture assays were performed in Methocult H4334 (Stem Cell Technologies, Vancouver, BC, Canada). Colonies were counted and scored on culture day 14.

Statistical analysis

Student's t-test was used to determine statistical significance for all the analyses in this study. Differences were defined as statistically significant when $P < 0{\cdot}05$.

Results

CD48 is constitutively expressed at high levels on almost all MM plasma cells, but at low levels on HSCs and HPCs

CD48 protein expression on CD38^{++} CD138$^+$ MM plasma cells and CD34$^+$ HSCs/HPCs was analysed by means of FACS in BM of MM patients (Fig 1A). CD48 was highly expressed on all CD38^{++}CD138$^+$ MM plasma cells, but only weakly on CD34$^+$CD38$^-$ HSCs and CD34$^+$CD38$^+$ HPCs. CD48 was highly expressed on more than 90% of the CD38^{++} plasma cells in 22 of the 24 MM patients whose BM was analysed (Fig 1B). In BM of MM patients, CD48^{++} cells overlapped with CD38^{++} plasma cells in most cases (Fig 1C).

CD48 expression levels on MM plasma cells were significantly higher than on normal leucocytes

In normal peripheral blood (PB), CD48 was expressed on CD19$^+$ B cells, CD3$^+$ T cells and SSC^{lo}CD13$^+$ monocytes. The expression levels of CD48 were compared between MM plasma cells and normal PB leucocytes by FACS (Fig 2A). CD48 expression levels on plasma cells were higher than those on lymphocytes or monocytes. Mean fluorescent intensities of MM plasma cells [1574 ± 727 (mean ± standard error (SE), $n = 3$)] were significantly ($P < 0{\cdot}05$) higher than those on normal T, B, and monocytes [137 ± 32, 197 ± 69, 291 ± 156, respectively ($n = 3$)] (Fig 2B). CD48 expression levels on granulocytes were lower than on lymphocytes and monocytes. Importantly, CD48 was not expressed on Glycophorin-A$^+$ erythrocytes or on CD41a$^+$ platelets.

The expression profiles of CD48 on BM cells from MM patients were compared with those of CD52, which is the target of alemtuzumab and known to be expressed extensively on mature leucocytes. Three MM samples were analysed and a representative finding is shown in Fig 2C. In all cases, both CD48 and CD52 were expressed on CD19$^+$ B cells, CD3$^+$ T cells, and CD13$^+$ myeloid cells, while no or only slight

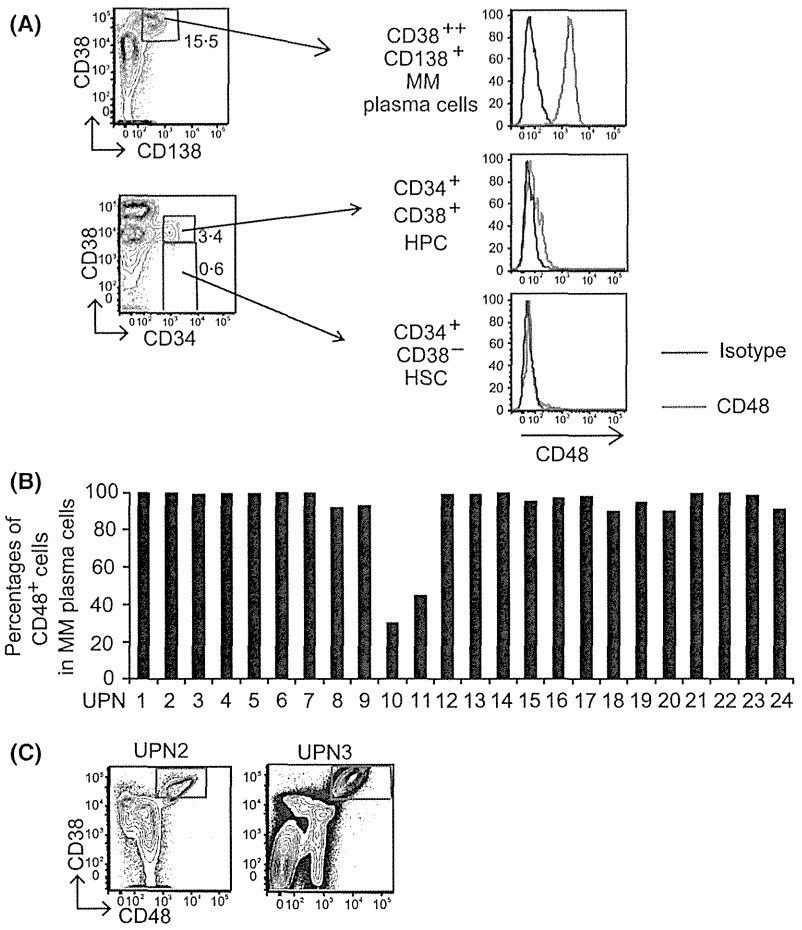

Fig 1. CD48 is constitutively expressed on MM plasma cells at much higher levels than on HSCs and HPCs. (A) Flow cytometric analysis of CD48 expression on $CD38^{++}$ $CD138^{+}$ MM plasma cells, $CD34^{+}CD38^{+}$ HPCs and $CD34^{+}CD38^{-}$ HSCs in BM of an MM patient (UPN1). (B) Percentages of $CD48^{+}$ cells in the $CD38^{++}$ $CD138^{+}$ plasma cell populations from MM patients. Each bar represents a single patient. UPN: unique patient number. (C) FACS analyses of CD38 and CD48 expression on BM cells from MM patients. Gates for $CD38^{++}CD48^{++}$ plasma cells are shown.

expression of the two molecules was detected on $CD34^{+}$ HSCs and HPCs. All $CD38^{++}$ MM plasma cells highly expressed CD48 but not CD52.

Novel anti-CD48 mAb can induce cytotoxicity against MM cells in vitro

Anti-human CD48 (hCD48) mAbs were prepared by immunizing Balb/c mice with hCD48-expressing mouse B cell line BaF3. Of three isolated hybridoma clones secreting anti-hCD48 mAbs, one with IgG2a isotype (clone 1B4) was subjected to further analysis, because the IgG2a isotype is reportedly preferable for ADCC and inhibiting tumour growth in xenograft models (Herlyn & Koprowski, 1982).

CD48 was expressed on all MM cell lines tested (OPM2, U266, MM1S, and RPMI8226) (Fig 3A). *In vitro* ADCC induced by the anti-CD48 mAb 1B4 was examined by using OPM2 and U266 as the targets and splenocytes of an SCID mouse as effector cells. ADCC induced by the anti-CD48 mAb against MM cell lines was weak but certainly detectable. By incubation with the anti-CD48 mAb and effector cells (E:T ratio = 50:1), 5·6 ± 0·8% of OPM2 and 10·8 ± 2·1% of U266 MM cells were killed, while background killing with the isotype control accounted for 2·5 ± 0·9% and 1·5 ± 1·6%, respectively (Fig 3B). The difference in cytotoxicity between the isotype control and the anti-CD48 mAb was statistically significant ($P < 0·05$). CDC induced by the anti-CD48 mAb against MM cell lines was very strong. By incubation with the anti-CD48 mAb and complement, 49·9 ± 0·7% of OPM2, 51·0 ± 7·3% of U266, 73·7 ± 1·9% of MM1S and 83·2 ± 12·6% of RPMI8226 cells were killed, while killing with the isotype control accounted for significantly ($P < 0·05$) lower percentages (Fig 3C). To examine whether lack of complement inhibitory protein was the reason for the high sensitivity of MM cell lines to the anti-CD48 mAb-induced CDC, expressions of CD55 and CD59 were examined. CD55/59 was variably expressed on all MM cell lines tested (Fig 3D). All MM cell lines were sensitive to the anti-CD48 mAb-induced CDC independently

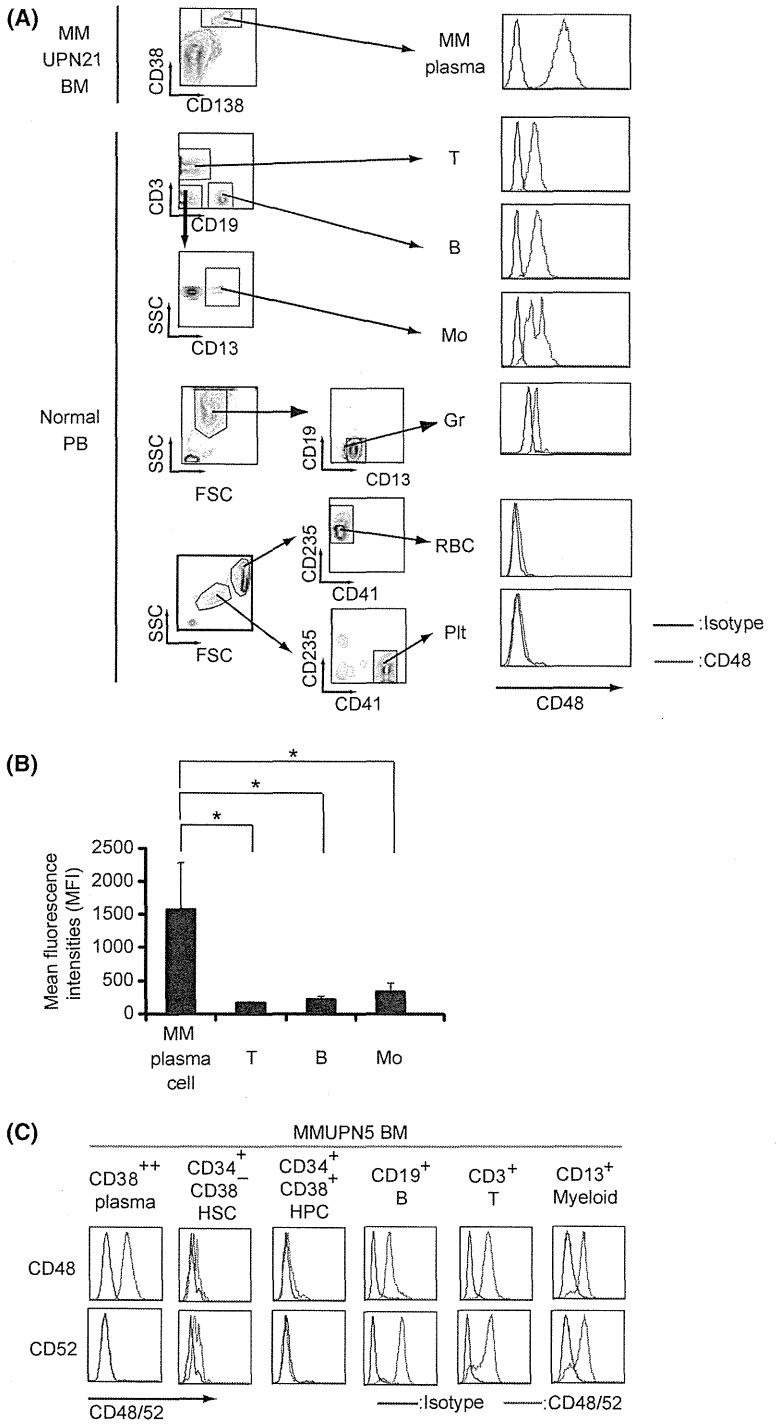

Fig 2. CD48 expression levels on MM plasma cells are much higher than those on normal leucocytes. (A) Flow cytometric analysis of CD48 expression on $CD38^{++}CD138^{+}$ plasma cells from a MM patient (UPN21), and $CD3^{+}$T cells, $CD19^{+}$B cells, $CD13^{+}SSC^{lo}$ monocytes (Mo), $CD13^{+}SSC^{high}$ granulocytes (Gr), $CD235^{+}$ Red blood cells (RBC) or $CD41^{+}$platelets (Plt) of peripheral blood (PB) cells from a healthy donor. Gating strategies for the subpopulations are shown on the left. (B) Mean fluorescent intensities (MFI) of CD48-fluorescein isothiocyanate (FITC) for plasma cells from MM patients, and T cells, B cells and monocytes from peripheral blood of healthy donors ($n = 4$). Bars represent standard errors (SE). *$P < 0{\cdot}05$. (C) Flow cytometric analysis of CD48 and CD52 expression in subpopulations of BM cells from an MM patient. Results of analysis of a representative MM patient (UPN5) are shown.

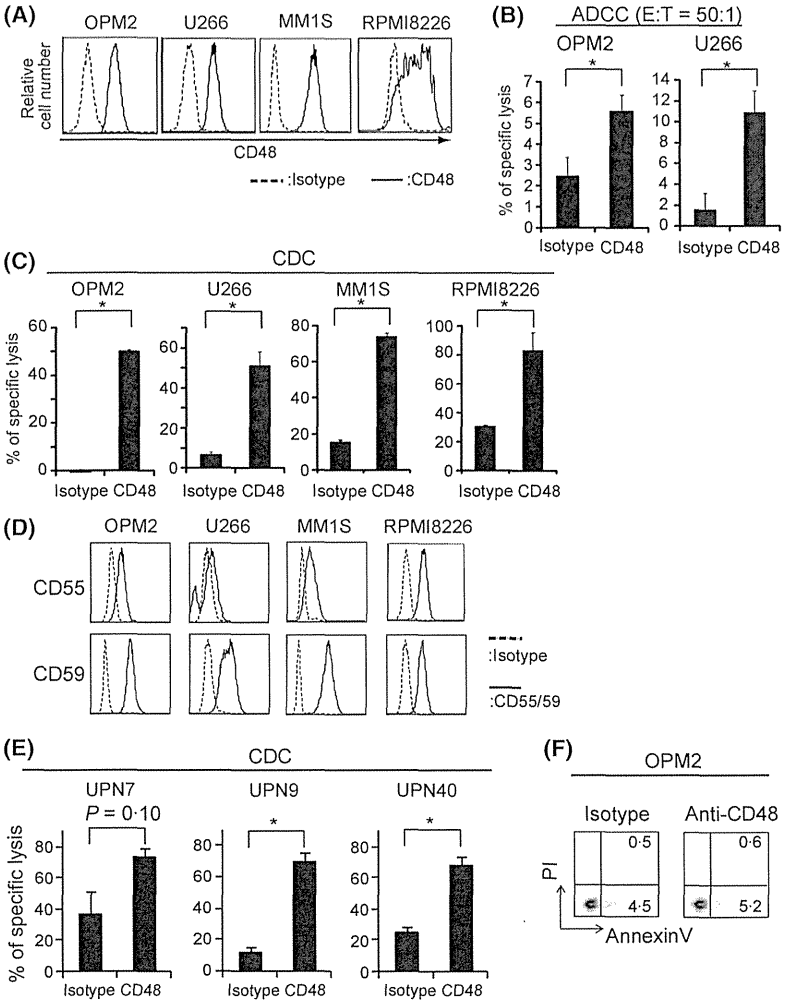

Fig 3. A new anti-CD48 mAb, 1B4, induces cytotoxicity against MM cells *in vitro*. (A) Flow cytometric analysis of CD48 expression on OPM2, U266, MM1S, and RPMI8226 MM cell lines. (B, C) Analysis of ADCC (B) and CDC (C) induced by administration of a new anti-CD48 mAb (1B4) using MM cell lines as target cells. The mean plus standard error (SE) of triplicate wells from one representative of three experiments is shown. $^*P < 0{\cdot}05$. E:T ratio in the ADCC analysis was 50:1. (D) Expression of CD55 and CD59 on MM cell lines. (E) 1B4-induced CDC against FACS-sorted MM plasma cells from patient samples. Error bars represent SEs of triplicate wells. $^*P < 0{\cdot}05$. (F) Annexin V/propidium iodide (PI) staining of OPM2 cells after incubation with the anti-CD48 mAb or isotype control, but without effector cells or complement, for 48 h.

of the expression levels of CD55 or CD59. Thus, loss of CD55 or CD59 is unlikely to be the reason for the high level of CDC activity of the anti-CD48 mAb against MM cell lines. We also performed a CDC assay with the anti-CD48 mAb against plasma cells purified from three MM patients, all of whom were resistant to chemotherapy and/or bortezomib. Anti-CD48 mAb-induced CDC killed 73·0 ± 10·0%, 69·3 ± 6·4% and 67·3 ± 5·9% of the MM plasma cells, while killing with the isotype control accounted for lower percentages (Fig 3E). Direct cytotoxic effects of the anti-CD48 mAb on MM cells without effector cells or complement were also examined. OPM2 MM cells were cultured in the presence of the anti-CD48mAb or isotype control for 48 h, followed by counting of apoptotic cells by means of Annexin V/propidium iodide staining. No significant difference in the number of Annexin V^+ cell was observed between treatment with anti-CD48mAb and that with the isotype control (Fig 3F).

In vivo anti-tumour activity of the anti-CD48 mAb in MM xenograft models

In vivo anti-MM activity of the anti-CD48 mAb 1B4 was tested in a subcutaneous MM tumour model, which has been used in many studies for testing the efficacy of mAb against MM (Tassone *et al*, 2004a,b; Yang *et al*, 2006; Tai *et al*, 2008). OPM2 MM cells were subcutaneously inoculated into SCID mice, which lacked T and B cells but had natural killer (NK) cell activity. Two weeks after tumour inoculation, mice with

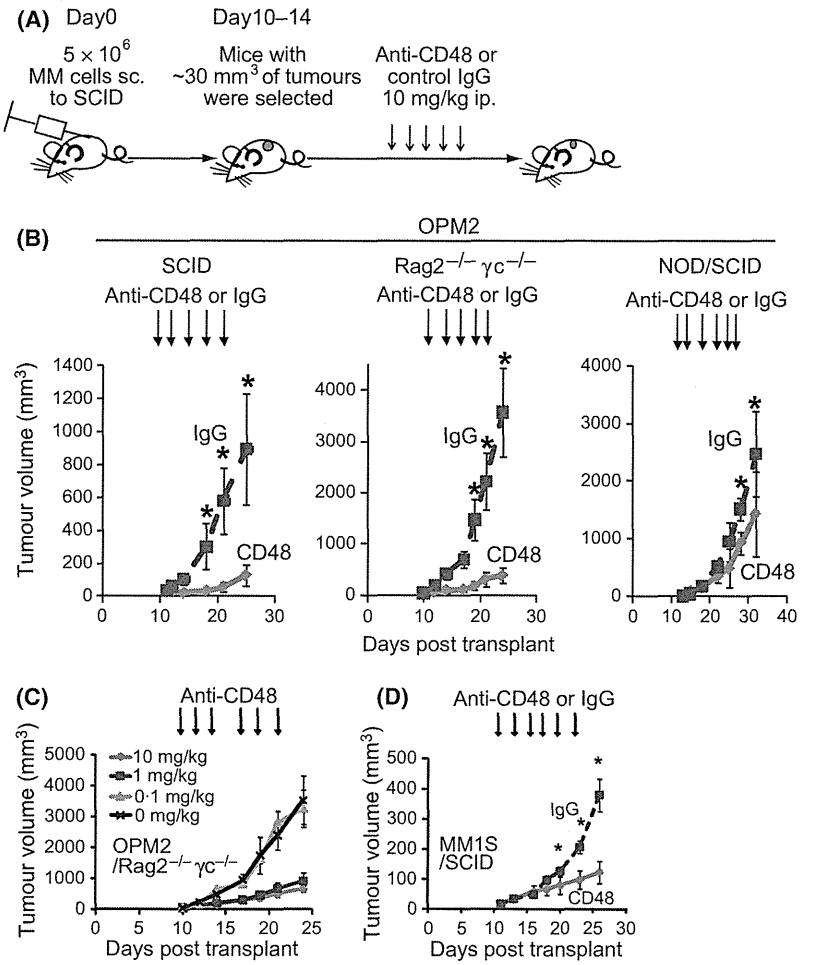

Fig 4. *In vivo* anti-tumour activity of the anti-CD48 mAb in subcutaneous MM xenograft models (A) Experimental design for anti-CD48 mAb treatment of severe combined immunodeficient (SCID) mice subcutaneously inoculated with MM cells. Mice with tumours were treated with the anti-CD48 mAb or control mouse IgG. (B) Tumour volumes of OPM2 cells in SCID, $Rag2^{-/-}\gamma c^{-/-}$, or non-obese diabetic (NOD)/SCID mice treated with the anti-CD48mAb or control mouse IgG ($n = 5$ for each type of mouse). Bars represent SE and asterisks show significant differences in tumour size ($P < 0{\cdot}05$). (C) $Rag2^{-/-}\gamma c^{-/-}$ mice inoculated with OPM2 MM cells were treated with different doses of the anti-CD48mAb (D) Tumour volumes of MM1S cells in SCID mice treated with either the anti-CD48 mAb or control IgG.

tumours approximately 30mm^3 in size were selected and treated three times a week with either the anti-CD48 mAb or control mouse IgG antibody (10 mg/kg body weight) for 2 weeks (Fig 4A). Tumour growth was significantly ($P < 0{\cdot}05$) inhibited in the mice treated with the anti-CD48 mAb, while in the mice treated with the control IgG antibody the tumour volume increased exponentially (Fig 4B). After 2 weeks of treatment, the average tumour volume was 136 ± 63 mm^3 in the anti-CD48 mAb-treated group ($n = 5$), but 897 ± 340 mm^3 in the control IgG-treated group ($n = 5$). $Rag2^{-/-}\gamma c^{-/-}$ mice that lacked T, B and NK cells were also used as recipients of subcutaneous OPM2 xenografts. Ten days after tumour inoculation, mice with tumours approximately 100 mm^3 in size were selected and treated three times a week with either the anti-CD48 mAb or the control mouse IgG antibody (10 mg/kg body weight) for 2 weeks. After 2 weeks of treatment, the average tumour volume was 380 ± 152 mm^3 in the anti-CD48 mAb-treated group ($n = 5$), but 3567 ± 857 mm^3 in the control IgG-treated group ($n = 5$) (Fig 4B). These results clearly show that the anti-CD48 mAb significantly ($P < 0{\cdot}05$) inhibited growth of MM *in vivo* in the absence of NK cells. To examine whether CDC is a major mechanism for MM growth inhibition by 1B4 mAb *in vivo*, OPM2 MM cells were also first inoculated in to NOD/SCID mice, which lacked not only lymphocyte but also complement activity, and then treated with 1B4 mAb. The inhibitory effect of 1B4 mAb was more prominent in SCID mice than in NOD/SCID mice (Fig 4B), suggesting that CDC is a major inhibitory mechanism. However, it should be noted that 1B4 mAb also significantly ($P < 0{\cdot}05$) reduced tumour growth in NOD/SCID mice, indicating that mechanisms other than CDC may be involved. The anti-MM effect of the anti-CD48mAb was found

to be dependent on the dosage of mAb (Fig 4C). Anti-tumour effect of the anti-CD48 mAb was also observed when MM1S MM cells were used as targets (Fig 4D). Taken together, these findings demonstrate that the anti-CD48 mAb is highly active for controlling growth of MM in murine xenograft models.

In vivo effect of the anti-CD48 mAb against MM cells inoculated into BM microenvironment

Since MM cells usually grow in BM but not in skin, the effect of the anti-CD48 mAb against MM cells that resided in a BM microenvironment was examined. OPM2 MM cells (3×10^5 cells) were directly injected into the left tibia of $Rag2^{-/-}\gamma c^{-/-}$ mice. Ten days after the injection, the chimerisms of human CD38 (hCD38)$^+$ OPM2 MM cells in BM of the left tibias were examined. This was followed by intravenous injection every other day of either the anti-CD48 mAb or control mouse IgG antibody at a dose of 5 mg/kg body weight. After the third mAb injection, the mice were sacrificed and examined for the chimerisms of hCD38$^+$ OPM2 MM cells in BM of the left tibias (Fig 5A). Human CD38$^+$ MM cells had decreased in three out of four mice treated with the anti-CD48 mAb, whereas MM cells in BM had expanded exponentially in all the mice treated with mouse IgG ($n = 4$) (Fig 5B, C). To exclude the possibility that MM cells had migrated to other sites from the injected bone, we investigated whether CD38$^+$ OPM2 MM cells could be detected in BM of the left femurs, right femurs and tibias, but no hCD38$^+$ MM cells were found. These results show that the anti-CD48 mAb treatment can inhibit growth of MM cells engrafted in a BM microenvironment.

Anti-CD48 mAb treatment did not induce CDC against CD34$^+$ HSCs/HPCs

The cytotoxic effect of the anti-CD48 mAb 1B4 against normal lymphocytes was investigated. *In vitro* CDC induced by the anti-CD48 mAb against CD8$^+$ T cells from a healthy individual was compared with that against the OPM2 MM cell line or purified MM plasma cells from a patient. Anti-CD48 mAb-induced CDC killed 74·2 ± 2·7% of the OPM2 cells, 77·8 ± 2·6% of patient plasma cells and 60·4 ± 0·1% of CD8$^+$ T cells, while background killing with isotype control antibody accounted for lower percentages (1·3 ± 0·0%, 36·2 ± 4·5% and 0·3 ± 0·3%) (Fig 6A). CDC against normal CD19$^+$ B cells was also tested. After incubation with the anti-CD48mAb and complement, 73·0 ± 5·8% of patient plasma cells and 67·8 ± 14·6% of CD19$^+$ B cells were killed, while background killing rates with isotype control antibody were lower (36·7 ± 14·3% and 44·0 ± 10·6%) (Fig 6A). These results indicate that normal T and B cells are also sensitive to *in vitro* CDC induced by the anti-CD48 mAb.

Weak CD48 expression was detected on CD34$^+$ HSCs/HPCs (Fig 1A). To test whether CD48 expression on CD34$^+$ HPCs caused CDC as a result of anti-CD48 mAb administration,

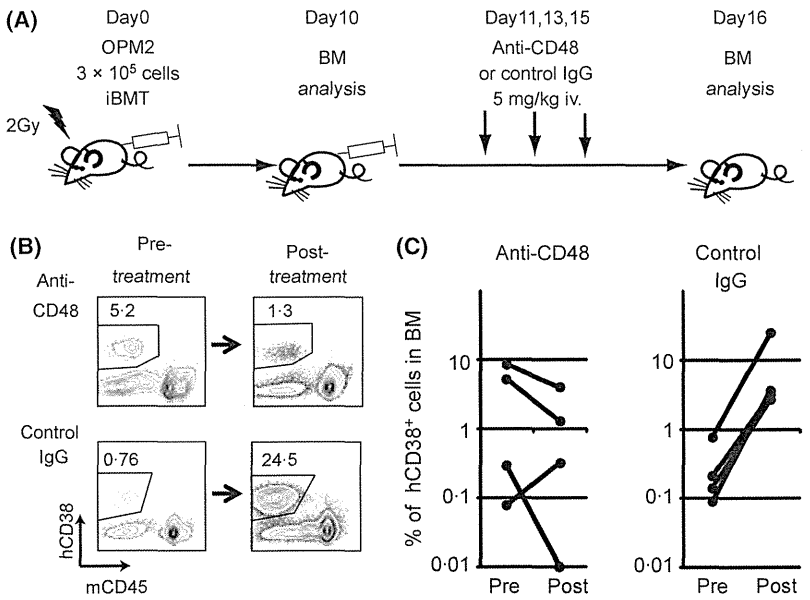

Fig 5. *In vivo* effect of the anti-CD48 mAb against MM cells inoculated in a BM microenvironment. (A) Experimental design for anti-CD48 mAb treatment of $Rag2^{-/-}\gamma c^{-/-}$ mice transplanted with OPM2 MM cells into bone marrow (BM) of the left tibia. iBMT denotes intra-bone marrow transplantation. Mice were intravenously inoculated with the anti-CD48 mAb ($n = 4$) or control IgG ($n = 4$). (B) Representative results of FACS analyses of BM cells from the left tibias on day 10 (pre-treatment) and day 16 (post-treatment). Human CD38 (hCD38)$^+$ cells represent OPM2 MM cells in mouse BM. (C) Chimerisms of hCD38$^+$ OPM2 MM cells in BM of the mice pre- and post-mAb treatment. Each dot corresponds to a treated mouse.

