Table 3 SVR and non-SVR in relation to the timing of HCV-RNA negativity in group A, B and C

	RVR $n = 38$	EVR $n = 9$	NVR $n = 1$	Total $n = 48$
RI>1.0 (Group A, $n = 9$)				
SVR (%)	1 (25)	0 (0)	0 (0)	1 (11)
Non-SVR (%)	3 (75)	4 (100)	1 (100)	8 (89)
RI ≤1.0, ≥3.7 log IU/mL* (Group B, $n = 23$)				
SVR (%)	18 (100)	5 (100)	0 (0)	23 (100)
Non-SVR (%)	0 (0)	0 (0)	0 (0)	0 (0)
RI ≤1.0, <3.7 log IU/mL* (Group C, $n = 16$)				
SVR (%)	16 (100)	0 (0)	0 (0)	16 (100)
Non-SVR (%)	0 (0)	0 (0)	0 (0)	0 (0)
Total ($n = 48$)				
SVR (%)	35 (92)	5 (56)	0 (0)	40 (83)
Non-SVR (%)	3 (8)	4 (44)	1 (100)	8 (17)

*HCV RNA loads at week 1.
SVR, sustained virologic responder; RVR, rapid virologic responder; EVR, early virologic responder; NVR, non-virologic responder; RI, rebound index.

RI below 1.0, all became HCV-RNA-negative within 8 weeks, thus achieving SVR status. The 24-week peginterferon plus ribavirin treatment for genotype 2 required the RI to be less than 1.0. Among the patients with a RI of ≤1.0, 16 had a viral load of less than 3.7 log IU/mL (Group C) at week 1. These patients were considered to be super-high responders to peginterferon. The early viral kinetics of these patients are shown in Table 4. The group included nine males and seven females with a mean age of 47.1 years. The mean age for men was 50.6 years, which

was higher than 42.8 years for women but the difference was not statistically significant. The viral load of these 16 patients before treatment was 5.90 log IU/mL, which was significantly lower than 6.22 log IU/mL, viral load for other SVR ($P < 0.01$). Of these 16, the viral load up to hour 24 was less than 3.70 log IU/mL in six patients. HCV-RNA was negative in week 2 in five of these six patients.

The viral loads of SVR patients in group A at baseline (RI >1.0), hour 24, weeks 1 and 2 were 6.08, 3.95, 4.40, and <3.70 log IU/mL, respectively. Compared with the viral load immediately before treatment, that at hour 24 was reduced by more than 2-log10.

Three patients interrupted treatment

Among the 51 patients who participated in the study, treatment was interrupted in three due to the development of adverse effects. These patients dropped out in weeks 5, 17, and 19. In these three patients, HCV-RNA became negative in week 4 and their RI was below 1.0. The patient who was discontinued in week 5 showed a relapse of HCV-RNA during a subsequent observation. The viral load for the two patients who dropped out in weeks 17 and 19 was less than 3.70 log IU/mL in week 1, and HCV-RNA continued to be negative 24 weeks after drug withdrawal. These two patients were judged to be SVR.

Predictive factors of SVR by multivariate analysis

Rebound index (≤1.0) was the only significant independent factor for SVR by multiple logistic regression analysis (Table 5). All other factors were not significant.

Table 4 Early viral kinetics of patients in super-high responder group (Group C)

Number	Age	Sex	HCV loads (log IU/mL)			HCV-RNA	
			Before	Hour 24	Week 1	Week 2	Week4
1	30	M	6.23	<3.70	<3.70	Negative	Negative
2	42	M	5.37	<3.70	<3.70	Negative	Negative
3	45	M	5.98	<3.70	<3.70	Negative	Negative
4	56	M	6.20	<3.70	<3.70	Negative	Negative
5	32	F	5.40	<3.70	<3.70	Negative	Negative
6	66	F	5.54	<3.70	<3.70	Positive	Negative
7	42	M	5.41	4.64	<3.70		Negative
8	48	M	6.08	4.28	<3.70		Negative
9	59	M	6.08	4.46	<3.70		Negative
10	66	M	6.28	4.23	<3.70		Negative
11	67	M	5.70	4.80	<3.70		Negative
12	30	F	6.18	4.41	<3.70		Negative
13	31	F	5.89	4.51	<3.70		Negative
14	32	F	5.94	4.08	<3.70		Negative
15	42	F	5.40	4.34	<3.70		Negative
16	67	F	5.70	4.63	<3.70		Negative

Factor	Category	Odds ratio	95% CI	P-value
Age	≥50 years	0.622	0.035–11.114	0.746
	<50 years	1		
Sex	Male	1.972	0.109–35.799	0.646
	Female	1		
BMI	<22.5	1.251	0.085–18.462	0.871
	≥22.5	1		
HCV load	<6.0 logIU/mL	0.98	0.061–15.788	0.988
	≥6.0 logIU/mL	1		
ALT	<50 IU/L	0.757	0.038–15.240	0.856
	≥50 IU/L	1		
Platelet count	≥18 × 10^4/mm^3	1.795	0.104–31.019	0.687
	<18× 10^4/mm^3	1		
Haemoglobin level	<14 mg/dL	0.398	0.012–12.7171	0.602
	≥14 mg/dL	1		
RI	≤1.0	689.586	4.214–>999.999	0.012
	>1.0	1		
Time to HCV RNA negativity(−)	≤Week 4	1.612	0.050–51.632	0.787
	>Week4	1		

BMI, body mass index; ALT, alanine aminotransferase.

DISCUSSION

The early viral kinetics in association with the peginterferon plus ribavirin treatment for genotype 1 have been reported [20,21]; but reports on early viral kinetics are scarce when the same combination is applied to genotype 2. This is the first investigation of early viral kinetics during peginterferon plus ribavirin therapy for genotype 2 chronic hepatitis C patients with high viral loads. We found that the RI (a new index) that is computed from the early viral kinetics is the first predictive factor for SVR as a substitute for RVR as a result of multiple analysis data. Patients with a RI of less than 1.0 and a viral load of less than 3.7 log IU/mL in week 1 were also identified as super-high responders to peginterferon plus ribavirin therapy.

The serum concentration of peginterferon alpha 2b peaked around 24 h, followed by a gradual decrease thereafter [22,23]. Thus the earlier studies on viral kinetics in association with peginterferon plus ribavirin for genotype 1 reported that the HCV load declines in hour 24 and increases again in week 1 [20,21]. In the responder group, the HCV load continues to decline every week thereafter [21]. A similar pattern is also seen in peginterferon monotherapy [22]. However, there are few reports on early viral kinetics involving genotype 2. In this study, the early viral kinetics of genotype 2 was investigated. Noting this increase in week 1, the viral load in week 1 was divided by that of hour 24 and the resultant coefficient was defined as the RI. In this study, the SVR rate was 100% for groups B and C, with the RI being less than 1.0. In these groups, HCV-RNA was eliminated by week 12 in all patients. On the other hand, re-emergence of virus was noted in 8% among the RVR. These

findings suggested that the RI is the first predictive factor for SVR as a substitute for RVR in 24-week peginterferon plus ribavirin therapy for genotype 2. For those patients with a RI of >1.0, treatment lasting more than 24 weeks appeared necessary.

Peginterferon plus ribavirin therapy results in SVR exceeding 80% in genotype 2 patients when treatment lasts for 24 weeks [1,2,9]. Because these patients are high responders to peginterferon plus ribavirin therapy, attempts have been made to shorten the duration of treatment [12–18]. Earlier, RVR patients have been treated for shorter periods (e.g. 12, 14 and 16 weeks) and it was reported that there was no difference in the SVR rate compared with the treatment duration of 24 weeks [12–15] According to a recent randomized study, the SVR rate is high even in RVR patients when treated for 24 weeks [16]. It has been reported that shortening of the treatment period results in economic advantages and reductions in the development of side effects [17]. Thus it becomes necessary to evaluate the super-high responder group to peginterferon plus ribavirin therapy who do not show reductions in the SVR rate even when the duration of treatment is reduced. Among those with genotype 2, about 80% or more convert to RVR but RVR alone does not sufficiently explain the state of super-high responders. In interferon therapy of genotype 2 patients, peginterferon alone produces therapeutic effects [2]. An HCV load below 3.0 log IU/mL on day 7 and undetectable HCV-RNA on day 29 were predictive of successful short-term treatment [18]. It is essential to identify super-high responders to peginterferon by using the early viral kinetics during the first 2 weeks of therapy. In this study, the viral load was investigated in week 1 following the

start of therapy, probably before the therapeutic effect of ribavirin manifests. Among 38 patients with RVR, 16 (group C) were found to have a viral load of less than 3.7 log IU/mL in week 1. It was believed that these patients constitute super-high responders to peginterferon; and that a high SVR rate may be reached when the duration of the peginterferon plus ribavirin therapy is curtailed to less than 12 weeks. The viral load of the two patients who had discontinued treatment in weeks 17 and 19 was less than 3.7 log IU/mL in week 1, and both converted to SVR after discontinuation of treatment. Further studies on a larger scale are needed.

CONCLUSION

Rapid virologic responder is a predictive factor for SVR in peginterferon plus ribavirin therapy. However, it was proven that the RI that was computed from the early viral kinetics in this study is the first predictive factor for SVR as a substitute for RVR by multiple logistic regression analysis. Patients with a RI less than 1.0 and a viral load of less than 3.7 log IU/mL (below the detectable level) in week 1 are also considered to be super-high responders to peginterferon plus ribavirin, thus constituting a group for whom the treatment period may be shortened. Further studies on a larger scale are necessary.

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Effective prediction of outcome of combination therapy with pegylated interferon alpha 2b plus ribavirin in Japanese patients with genotype-1 chronic hepatitis C using early viral kinetics and new indices

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Abstract

Background The rates of sustained virologic response (SVR) and relapse with pegylated interferon alpha 2b (peginterferon) plus ribavirin in patients with genotype-1 chronic hepatitis C (CHC) are approximately 50 and 30%, respectively. We investigated whether SVR and transient response (TR) can be differentiated during treatment using new indices calculated from early viral kinetics and the timing of when hepatitis C virus (HCV)-RNA becomes undetectable.

Methods Peginterferon alpha 2b (1.5 μg/kg per week) plus weight-based ribavirin (600–1,000 mg/day) were administered to 141 patients with genotype-1 CHC for 48 weeks. The HCV-RNA loads were measured at baseline, 24 h, week 1, and week 2. The rebound index (RI, viral load at week 1 divided by viral load at 24 h) and the second rebound index (RI-2nd, viral load at week 2 divided by viral load at 24 h) were calculated.

Results With SVR, the viral load was reduced at 24 h, did not rise during week 1 (RI ≤ 1.0), and was significantly reduced at week 2 ($P < 0.05$). Viral loads with TR and non-response increased at week 1. The SVR rate was 90% with RI ≤ 1.0, 96% with rapid viral responders, and 93% with RI-2nd <0.7 and week 8 early viral responders. The SVR rate with these 3 groups was 90% and administration for 48 weeks was recommended. With other groups, the SVR rate was 23% and the TR rate was 77%. Administration for 72 weeks was therefore recommended.

Conclusions We distinguished SVR from TR during treatment using two indices (RI and RI-2nd) and the timing of HCV-RNA negativity.

Abbreviations

SVR	Sustained virologic response
TR	Transient response
NR	Non-response
RI	Rebound index
RI-2nd	Second rebound index
RVR	Rapid viral responder
W8EVR	Week 8 early viral response
W12EVR	Week 12 early viral response
LVR	Late viral responder
NVR	Non-viral responder

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Introduction

The first choice of treatment of genotype 1 chronic hepatitis C (CHC) is combination therapy with pegylated interferon alpha 2b (peginterferon) and ribavirin. The duration of treatment for genotype 1 is 48 weeks [1, 2]. Factors predictive of sustained virologic response (SVR) to

peginterferon plus ribavirin include genotype, viral load, age, histology, and amino acid substitutions in the hepatitis C virus (HCV) [3–6]. None of these predictive factors is adequate in predicting SVR in patients with genotype 1 and a high viral load prior to treatment.

With the current standard of care of peginterferon plus ribavirin administered for 48 weeks, the SVR rate in patients with genotype 1 and a high viral load is about 50% [1, 2]. It has been recognized, however, that the SVR rate increases if the duration of treatment is extended to 72 weeks [7–9]. The result of treatment with peginterferon plus ribavirin is SVR, transient response (TR), or non-response (NR). At the end of treatment, about 80% of patients are HCV RNA-negative, but about 30% of these patients relapse (TR) after the end of treatment, resulting in an actual SVR rate of about 50%. To increase the SVR rate, this incidence of relapse must be reduced; to achieve this, the duration of treatment needs to be extended to 72 weeks for patients who may potentially relapse after the end of treatment [7]. Differentiation between SVR and TR is therefore essential during treatment.

HCV RNA negativity status at weeks 4 and 12 during treatment is important in predicting SVR [6, 10–12], with reduction in the SVR rate observed if HCV RNA is not undetectable by week 12. In other words, for the early determination of the therapeutic efficacy of peginterferon plus ribavirin treatment, HCV RNA negativity by week 4 (rapid viral responder: RVR), HCV RNA negativity by week 12 (early viral responder: EVR), and HCV RNA negativity by week 24 (late viral responder: LVR) are considered important. EVR is a better predictor of SVR than the predictive factors that can be determined prior to treatment. EVR is therefore considered to be an index of therapeutic effect in the early stage of peginterferon plus ribavirin treatment. In a recent trend, a duration of treatment of 72 weeks is being selected when HCV RNA is detected at week 12 but is undetectable at week 24. However, distinguishing SVR from TR during treatment is difficult by these two time points when HCV RNA is not detected.

For a more accurate determination of SVR and TR during treatment, HCV RNA was examined at week 8 in addition to weeks 4, 12, and 24, and the SVR rate was examined based on HCV RNA negativity at these time points. Early viral kinetics up to week 2, considered to be the index of the therapeutic effect of peginterferon alone, were also evaluated and two new indices were defined. Distinguishing SVR from TR during peginterferon treatment was possible by combining these new indices and the timing of HCV RNA negativity and allowed the assignment of patients to 48- or 72-week treatment as a result.

Patients and methods

A total of 149 patients with genotype 1 CHC were treated with peginterferon plus ribavirin at the Shin-Kokura Hospital between December 2004 and May 2006. Of these patients, treatment was interrupted in 8 patients, so this study was conducted on the remaining 141 patients who completed 48 weeks of treatment. Eighty were male and 61 were female, with ages ranging from 27 to 70 years (mean: 53.2 ± 10.8), and 109 individuals were naïve to interferon therapy. The viral load at enrollment exceeded 100,000 IU/ml. The results of liver biopsy conducted within 6 months of enrollment confirmed chronic hepatitis (F1-F3), and diagnosis was based on the scoring system of Desmet et al. [13]. All patients received 1.5 μg/kg of peginterferon alpha-2b (PegIntron, Schering-Plough, Osaka, Japan) administered subcutaneously once a week in combination with ribavirin (Rebetol, Schering-Plough, Osaka, Japan) administered orally at a daily dose of 600–1,000 mg based on body weight (600 mg for patients weighing less than 60 kg, 800 mg for those weighing 60–80 kg, and 1,000 mg for those weighing more than 80 kg).

Peginterferon was administered at 9:00 in the morning for the initial, second, and third doses. The HCV loads were measured immediately before the start of treatment, at 24 h post-dose, and at weeks 1 and 2. The coefficient derived by dividing the viral load at week 1 by that at 24 h was defined as the rebound index (RI), while the coefficient derived by dividing the viral load at week 2 by that at 24 h was called the second rebound index (RI-2nd). The patients were divided into the following 3 groups based on RI and RI-2nd: RI-A group (RI $\leq$ 1.0), RI-B group (RI > 1.0 and RI-2nd < 0.7), and RI-C group (RI > 1.0 and RI-2nd $\geq$ 0.7).

The qualitative test for HCV RNA was conducted 6 times (at weeks 4, 8, 12, and 24, at the end of treatment, and at week 24 after the end of treatment). Patients who were HCV RNA-negative by week 4 were considered rapid viral responders (RVR), patients who were HCV RNA-negative between weeks 5 and 12 were considered early viral responders (EVR), and patients HCV RNA-negative between weeks 13 and 24 were considered late viral responders (LVR). EVR was further divided into week 8 EVR (HCV RNA-negative between weeks 5 and 8, W8EVR) and week 12 EVR (HCV RNA-negative between weeks 9 and 12, W12EVR). Patients HCV RNA-positive at week 24 were considered non-viral responders (NVR). Patients who remained HCV RNA-negative up to 24 weeks after the end of treatment were considered to have achieved SVR. Patients HCV RNA-negative by week 24 of treatment but who became positive again after the end of treatment were considered TR. Patients who failed to achieve HCV RNA negativity by the end of treatment were

considered NR. None of these patients were HCV RNA-negative between weeks 25 and 48.

Sera were collected from the patients before and during treatment and frozen for determination of viral loads by a quantitative HCV RNA PCR assay (COBAS Amplicor HCV Monitor Test v2.0 using a 10-fold dilution method, Roche Diagnostics, Tokyo, Japan), which has a low threshold of quantitation of 5,000 IU/ml and an outer limit of quantitation of 5,100,000 IU/ml. A qualitative test for serum HCV RNA was performed using Amplicor-HCV kit version 2.0 (Roche Diagnostics, Tokyo, Japan) and the results were labeled positive or negative. The lower limit of detection was 50 IU/ml. All testing was performed at a single reference laboratory. The HCV genotype was determined by a type-specific primer from the core region of the HCV genome. Genotyping was carried out as described previously [14].

Criteria for exclusion were [1] clinical or biochemical evidence of hepatic decompensation and advanced cirrhosis identified by ascites, encephalopathy, or hepatocellular carcinoma [2], white blood cell count of less than 3,000/mm³ and platelet count of less than 50,000/mm³ [3], concurrent liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive), [4] excessive active alcohol consumption over 60 g/day or drug abuse, [5] severe psychiatric disease, or [6] antiviral or corticosteroid therapy within the 12 months prior to enrollment. Both peginterferon alpha-2b and ribavirin were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 8.5 g/dl, 1,000/mm³ and 25,000/mm³, respectively. Treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic problems developed, if continuation of treatment was judged not to be possible by the attending physician, or if the patient no longer desired to continue treatment.

Informed consent

The study protocol was approved by the Institutional Ethics Committee of Shin-Kokura Hospital, and all patients gave informed consent to participate in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice.

Statistical analysis

Differences between viral loads between two groups were analyzed using the Student's t test and Mann–Whitney rank-sum test. We conducted analysis using the Kruskal Wallis test for three-group (SVR, TR, and NR) and five-group (RVR, W8EVR, W12EVR, LVR and NVR) comparisons. All statistical analyses were conducted on a Macintosh computer using StatView 5.0 (Abacus Concepts, Berkeley, CA, USA). P values of <0.05 were considered to be statistically significant.

Results

Baseline characteristics of patients grouped by SVR, TR and NR

SVR was observed in 72 patients (51.1%), TR in 40 patients (28.4%), and NR in 29 patients (20.6%). The characteristics at enrollment of patients showing SVR, TR, or NR are presented in Table 1. The mean age of SVR, TR, and NR patients was 50.8, 56.6, and 56.0 years, respectively. There were no significant inter-group differences in mean age, gender, or pre-treatment test results (alanine aminotransferase, hemoglobin level, platelet count, and viral load).

Table 1 Baseline characteristics of patients by response (SVR, TR, and NR)

	SVR $n = 72$	TR $n = 40$	NR $n = 29$	Total $n = 141$	P value[a]
Age (years)	50.8 (11.3)	56.6 (8.6)	56 (10.4)	53.2 (10.8)	0.084
Male (%)	41 (56)	24 (52)	19 (65)	84 (57)	
Laboratory					
ALT (IU/l)	88 (82)	86 (53)	94 (78)	90 (74)	0.808
Hemoglobin level (g/dl)	14.5 (1.4)	14.6 (1.3)	14.6 (1.0)	14.5 (1.3)	0.917
Platelet count (×10⁴/mm³)	19 (6)	17 (6)	20 (10)	19 (7)	0.707
HCV RNA loads (×10³ IU/ml)	2299 (1634)	2228 (1344)	2390 (1501)	2298 (1524)	0.953
Body mass index (kg/m²)	23.6 (5.7)	24.8 (2.9)	23.9 (3.6)	24 (4.9)	0.536

Values are represented as means with standard deviation in parentheses or as absolute values with percentages in parentheses

SVR sustained virologic response, TR transient response, NR non response, ALT alanine aminotransferase

[a] Kruskal Wallis Test

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341

Early viral kinetics, RI and RI-2nd relative to SVR, TR and NR

Viral kinetics up to the first two weeks after the start of treatment are shown for the SVR, TR, and NR groups (Fig. 1 and Table 2). The viral load at 24 h for the SVR and TR groups (226,000 and 229,000 IU/ml) was reduced significantly compared to the NR group (523,000 IU/ml) ($P \leq 0.05$). The differences of the viral loads for three groups at weeks 1 and 2 were significant ($P \leq 0.0001$). The viral load of the SVR group at weeks 1 and 2 was significantly lower than that of the TR group ($P < 0.01$ and $P < 0.05$). The former was reduced at week 1 with no increases thereafter, and the viral load at week 2 (76,000 IU/ml) was significantly lower than at 24 h

Fig. 1 HCV-RNA kinetics during the first 2 weeks of treatment by SVR (*black circle*), TR (*black triangle*), and NR (*black square*). a $P < 0.05$, b $P < 0.0001$, c $P < 0.0001$ (Kruskal Wallis test), d $P < 0.01$ (hour 24 vs. week 2 in SVR). *SVR* sustained virologic response, *TR* transient response, *NR* non-response

(226,000 IU/ml, $P < 0.01$). The viral load of the TR group rose to 397,000 IU/ml at week 1 and was reduced to 169,000 IU/ml at week 2. This reduction was not significant when compared against that at 24 h (229,000 IU/ml). The viral load of the NR group rose again to 1,206,000 IU/ml at week 1 and was reduced to 615,000 IU/ml at week 2, which was still higher than that at 24 h (523,000 IU/ml).

RI and RI-2nd for the SVR, TR and NR groups are shown in Table 2. RI for the SVR group (0.8) was below 1.0. The differences of the RI for the three groups were significant ($P \leq 0.0001$). RI-2nd for the SVR, TR and NR groups was 0.3, 0.8, and 1.3, respectively, with the highest value observed with the NR group. The differences of the RI-2nd for three groups were significant ($P \leq 0.0001$). RI-2nd for the TR group was significantly higher than for the SVR group ($P < 0.05$).
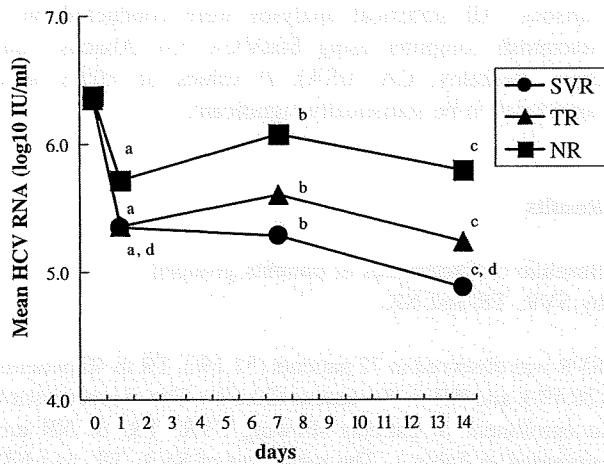
SVR rates, early viral kinetics, RI and RI-2nd relative to the timing of HCV RNA negativity

RVR, W8EVR, W12EVR, LVR, and NVR were observed in 26 (18.4%), 31 (22.0%), 31 (22.0%), 24 (17.0%), and 29 (20.6%), respectively The SVR rate with the RVR, W8EVR, W12EVR, and LVR groups was 96.2% (25/26), 83.9% (26/31), 54.8% (17/31), and 16.7% (4/24), respectively. None in the NVR group exhibited the absence of HCV-RNA at the end of treatment. The HCV RNA kinetics for the RVR, W8EVR, W12EVR, LVR, and NVR groups up to week 2 of treatment are shown in Fig. 2 and Table 3. The viral load of the RVR group was rapidly reduced to 143,000 IU/ml by 24 h, with a further drop to 55,000 IU/ml at week 1. At week 2, the viral load was reduced to 8,000 IU/ml, which was significantly less than that at 24 h ($P < 0.001$). The viral loads for the W8EVR and W12EVR groups were reduced to 186,000 IU/ml and 134,000 IU/ml, respectively, by 24 h but rose to 231,000 IU/ml and

Table 2 Kinetics of HCV RNA during the first 2 weeks of treatment by response (SVR, TR, and NR)

	SVR ($n = 72$)		TR ($n = 40$)		NR ($n = 29$)		P value[a]
	Mean	SD	Mean	SD	Mean	SD	
HCV loads ($\times 1000$ IU/ml)							
Before treatment	2299	(1634)[b]	2228	(1344)	2390	(1501)	0.9538
Hour 24	226	(328)	229	(249)	523	(518)	0.0102
Week 1	190	(302)	397	(399)	1206	(811)	<0.0001
Week 2	76	(193)[b]	169	(249)	615	(617)	<0.0001
Rebound index	0.8	(0.9)	2.9	(3.1)	3.1	(1.8)	<0.0001
Rebound index 2	0.3	(0.6)	0.8	(0.7)	1.3	(1.1)	<0.0001

Values represent means with ranges in parentheses

[a] Kruskal Wallis Test

[b] $P < 0.01$ (hour 24 vs. week 2 in SVR)

Abbreviations *SVR* sustained virologic response, *TR* transient response, *NR* non response, *SD* standard deviation

277,000 IU/ml, respectively, at week 1. The viral load of the W8EVR group at week 2 was reduced to 49,000 IU/ml, which was significantly less than that at 24 h ($P < 0.001$). The viral load of the W12EVR group, on the other hand, was 119,000 IU/ml, which was not significantly less than that at 24 h. The viral load of the LVR group at 24 h (563,000 IU/ml) was higher than that of RVR, W8EVR, or W12EVR. The viral load rose further to 674,000 IU/ml at week 1, and although a reduction to 361,000 IU/ml was observed at week 2, it was still significantly greater than with RVR, W8EVR, and W12EVR ($P < 0.001, P < 0.001$, and $P < 0.01$, respectively). The viral load (523,000 IU/ml) of the NVR group at 24 h was similar to that of the LVR group. It rose after one week and was not low in the second week (615,000 IU/ml) compared to that at 24 h. The differences of the viral loads for five groups were significant at hour 24, week 1, or week 2 ($P \leq 0.0001$).

RI and RI-2nd of the RVR, W8EVR, W12EVR, LVR, and NVR groups are shown in Table 3. RI of RVR (0.4) was the lowest and was lower than that of W8EVR (2.3), W12EVR (2.4), LVR (1.5), and NVR (3.1). RI-2nd of NVR (1.3) was the highest, being higher than RVR (0.1), W8EVR (0.5), W12EVR (0.7), and LVR (0.7) ($P < 0.001, P < 0.01, P < 0.05$, and $P < 0.01$, respectively). The mean RI-2nd for other than NVR was below 0.7. The differences of the RI or RI-2nd for five groups were significant ($P \leq 0.0001$).

SVR, TR, and NR rates relative to RI and RI-2nd

The 3 groups (RI-A, RI-B, and RI-C) and 4 groups (RVR, W8EVR, W12EVR, and LVR) were combined and then divided into 12 groups, with SVR, TR, and NR grouped by
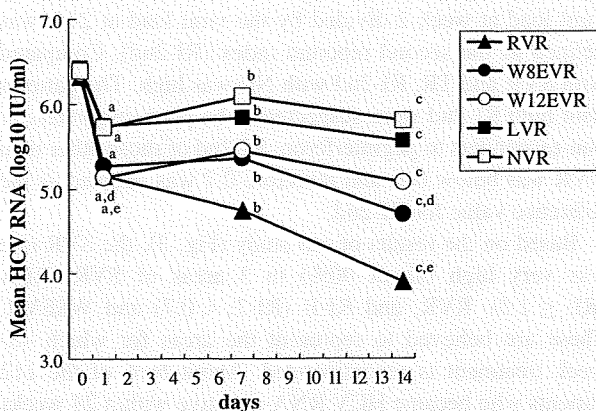

Fig. 2 HCV RNA kinetics by RVR (*black triangle*), W8EVR (*black circle*), W12EVR (*white circle*), LVR (*black square*), and NVR (*white square*) during the first 2 weeks of treatment a $P < 0.001$, b $P < 0.0001$, c $P < 0.0001$ (Kruskal Wallis test), d $P < 0.001$ (hour 24 vs. week 2 in W8EVR), e $P < 0.001$ (hour 24 vs. week 2 in RVR). *RVR* rapid viral response, *W8EVR*, week 8 early viral response, *W12EVR* week 12 early viral response, *LVR* late viral response, *NVR* non-viral response

RI and RI-2 and by RVR, W8EVR, W12EVR, and LVR (Fig. 3). The SVR, TR, and NR rates were 90.2% (46/51), 9.8% (5/51), and 0% (0/51), respectively, with RI-A (RI $\leq$ 1.0), 55.6% (25/45), 40.0% (18/45), and 4.4% (2/45), respectively, with RI-B (RI > 1.0, RI-2nd < 0.7), and 2.2% (1/45), 37.8% (17/45), and 60.0% (27/45), respectively, with RI-C (RI > 1.0, RI-2nd $\geq$ 0.7). The SVR rate for RI-A and RVR was 90.2% (46/51) and 96.2% (25/26), respectively. The SVR rate for RI-B and W8EVR was 93.3% (14/15). The SVR rate for the patients in these 3 areas was 89.7% (61/68), suggesting that they represent the population for which 48-week treatment is appropriate. Among the 112 patients who became HCV RNA-negative at week 24, 60.7% (68/112) were in the above 48-week regimen area. In particular, the SVR rate (2.2%) from RI-C was very low and the TR and NR rates were 37.8% and 60.0%, respectively. Among W8EVR, W12EVR, and LVR in the range outside that for a 48-week regimen, the SVR rate (25.0%, 11/44) was low but the TR rate (75.0%, 33/44) was high. Thus extension of the treatment period was considered necessary, and a 72-week regimen was recommended.

Discussion

This is the first study in which SVR, TR, and NR, in response to 48 weeks of peginterferon plus ribavirin treatment, were successfully distinguished in the early stage of treatment. This was possible by using new indices (rebound index: RI, and second rebound index: RI-2nd) calculated from early viral kinetics and the timing of when HCV RNA becomes undetectable. This allows for the TR group to be treated for 72 weeks, potentially raising the SVR rate.

In the treatment of genotype 1 CHC with peginterferon plus ribavirin, relapse occurs in about 30% of patients after the end of treatment [1, 2]. In LVR (late viral responders), in particular, the percentage of relapse is high (59%) after 48 weeks of treatment [7]. It is vital to reduce the relapse rate (TR rate) and raise the SVR rate. This requires (1) dose increase and (2) prolongation of the period of treatment. In Japanese patients, the dose of peginterferon must often be reduced because of the onset of such adverse events as neutropenia, thrombocytopenia, and malaise, and thus dose increase is not a feasible option. The dose of ribavirin must also be reduced in some patients due to ribavirin-induced anemia, and likewise, any increase in dose is not feasible [15]. Thus extending the duration of treatment to 72 weeks is considered necessary, and it becomes essential to distinguish the population for which 48-week treatment is adequate from the population for which 72-week treatment is necessary. In a previous study, HCV RNA-negativity was determined at weeks 12 and 24 [7]. SVR was noted in

Table 3 Kinetics of HCV RNA during the first 2 weeks of treatment by response (RVR, W8EVR, W12EVR, LVR, or NVR)

	RVR (n = 26)		W8 EVR (n = 31)		W12 EVR (n = 31)		LVR (n = 24)		NVR (n = 29)		P value[a]
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
HCV loads (×1000 IU/ml)											
Before	2102	(1636)[b]	2271	(1536)[c]	2259	(1744)	2625	(1278)	2390	(1501)	0.9172
24 h	143	(182)	186	(191)	134	(95)	563	(558)	523	(518)	0.0007
1 week	55	(66)	231	(213)	277	(325)	674	(503)	1206	(811)	<0.0001
2 week	8	(5)[b]	49	(86)[c]	119	(264)	361	(299)	615	(617)	<0.0001
Rebound index	0.4	(0.3)	2.3	(3.6)	2.4	(2.8)	1.5	(0.6)	3.1	(1.8)	<0.0001
Rebound index 2	0.1	(0.1)	0.5	(0.6)	0.7	(1.4)	0.7	(0.4)	1.3	(1.1)	<0.0001

RVR rapid viral response, W8EVR week 8 early viral response, W12EVR week 12 early viral response, LVR late viral response, NVR non viral response, SD standard deviation

[a] Kruskal Wallis Test

[b] P < 0.001 (hour 24 vs. week 2 in RVR)

[c] P < 0.001 (hour 24 vs. week 2 in W8 EVR)

18% even when treatment was continued to week 48 in patients who were HCV RNA-positive at week 12 but HCV RNA-negative at week 24 (LVR) [7]. If treatment is continued for 72 weeks, these patients will receive drugs unnecessarily for an extra 24 weeks. On the other hand, in this study, the SVR rate with W8EVR and W12EVR was 84% and 55%, respectively. The SVR rate for the overall EVR (W8EVR + W12EVR) was 69%, with a relapse rate of 31%. By treating these relapsed patients for 72 weeks, a higher SVR can be expected.

For more effective treatment with peginterferon plus ribavirin, indices besides the currently used index (the time to HCV RNA negativity) should be introduced and evaluated. In this study, we succeeded in differentiating the populations to be treated for 48 and 72 weeks more accurately by measuring the viral loads up to week 2 after the start of treatment and calculating two new indices (rebound index and second rebound index). Unrelated to ribavirin, lowering the dose of peginterferon at the early stage of treatment reduces the SVR rate [16]. In other words, the therapeutic effect of peginterferon, independent of that of ribavirin at the early stage of treatment, is expected to be responsible for SVR and EVR, which is believed to occur before ribavirin takes effect. Early viral kinetics were determined up to week 2, which are believed to express the therapeutic effect of peginterferon. The serum concentration of peginterferon alpha 2b peaks after 24 h, followed by a gradual decline [17, 18]. The viral load is therefore reduced by 24 h but increases in week 1 [19, 20]. A large dose of peginterferon at each administration results in a marked reduction in the viral load at 24 h but the viral load increases in week 1 regardless of the dose. In the responder group, the viral load continues to decline each week thereafter [20]. This trend is also seen with peginterferon monotherapy [17]. On the other hand, in the SVR group, in particular the RVR

group, it was noted that a number of patients did not experience an increase in the viral load at week 1. As shown in Fig. 1, in SVR, the viral load does not increase at week 1, while a return of viral loads is seen in TR and NR. The viral loads of SVR and TR were lower than that of NR at week 2. The viral load in week 1 divided by the viral load at 24 h was therefore defined as the rebound index (RI). The RI of SVR is 0.8 (less than 1.0), which is less than that for TR or NR. Among the RI of RVR, W8EVR, W12EVR, LVR, and NVR, only that of RVR was below 1.0. Among the 26 RVR patients, 24 (92%) exhibited RI-A (RI: ≤ 1.0) without a rise in week 1 (Fig. 3). The SVR rate with RI-A was 90%. It was believed that this group (RI-A, RI: ≤ 1.0) was composed of high responders to peginterferon. Because no decline in the viral load is noted in non-responders after week 2 [20], the viral load at week 2 divided by the viral load at 24 h was defined as the second rebound index (RI-2nd). Compared with SVR and TR, RI-2nd with NR was high. The patients with high RI-2nd were suspected to be poor responders or non-responders to peginterferon. RI-2nd of those other than NVR was below 0.7; and therefore 0.7 was adopted as the reference value for RI-2nd.

Based on the results of our study (Fig. 3), the SVR rate was very high (about 90%) in 3 areas of SVR: RI-A (RI: ≤ 1.0), RVR, and RI-B (RI-2: < 0.7) and W8EVR. These are believed to represent the areas for which 48-week treatment is recommended. About 60% of the 112 patients who became HCV RNA-negative within 24 weeks were in this area, and the SVR rate of the remaining 40% was low (25%) while the TR rate was high (75%). It was therefore thought that 72-week treatment is needed for these patients.

In peginterferon and ribavirin treatment, the status of EVR is important. When HCV RNA-negativity is not achieved by week 12, the SVR rate becomes very low [10].

Fig. 3 SVR, TR, and NR by RI and RI-2nd as well as RVR, W8EVR, W12EVR, LVR, and NVR. *SVR* white circle, *TR* white triangle, *NR* white square, *SVR* sustained virologic response, *TR* transient response, *NR* non-response, *RI* rebound index, *RI-2nd* rebound index second, *RVR* rapid viral response, *W8EVR* week 8 early viral response, *W12EVR* week 12 early viral response, *LVR* late viral response, *NVR* non-viral response

	RVR N=26	W8EVR N=31	W12EVR N=36	LVR N=26	NVR N=29	Total N=148
RI>1.0, RI-2nd≥0.7 RI-C, N=52		△△△	△△△△ ○△△△△△	△△△△△△ ○△△△△△	□□□ □□□□□□ □□□□□□ □□□□□□ □□□□□□	SVR:3.9% TR:44.2% NR:51.9%
RI>1.0, RI-2nd<0.7 RI-B, N=45	○△	○○△ ○○○○○○ ○○○○○○	△△ ○△△△△△ ○○○○○○	△△△△△△ ○○○△△△	□□	SVR:55.6% TR:30.0% NR:4.4%
RI≤1.0 RI-A, N=51	○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○	△ ○○○○○○ ○○○○○○	○○○△△△ ○○○○○○	○△		SVR:90.2% TR:9.8% NR:0.0%
Total N=148	SVR:96.2% TR:3.8% NR:0.0%	SVR:83.9% TR:16.1% NR:0.0%	SVR:47.2% TR:52.8% NR:0.0%	SVR:19.2% TR:80.8% NR:0.0%	SVR:0.0% TR:0.0% NR:100%	SVR:49.3% TR:31.1% NR:19.6%

In our study, SVR was low (below 20%) among the LVR who became negative for HCV RNA between weeks 12 and 24. Mangia et al. reported that to raise the SVR rate, treatment for 48 weeks is needed if HCV RNA becomes negative at week 8, while treatment for 72 weeks is needed if HCV RNA negativity is observed at week 12 [21]. In our study, the SVR rate with RVR was 96% and was also very high (84%) with W8EVR achieving HCV RNA negativity between 5 and 8 weeks. On the other hand, the SVR rate was low (55%) in patients who became HCV RNA-negative between weeks 9 and 12. These findings suggested that in treating Japanese patients with CHC with peginterferon plus ribavirin for 48 weeks, EVR should be qualified at week 8 rather than at week 12. Therefore, for evaluation, EVR patients who became HCV RNA-negative by week 8 were classified as W8EVR and those who became HCV RNA-negative by week 12 were classified as W12EVR. Early viral kinetics of both W8EVR and W12EVR indicated a rebound at week 1 but the viral load of W8EVR at week 2 was significantly lower than that at 24 h. On the other hand, the reduction in the viral load of W12EVR at week 2 was not significant when compared against that at 24 h. A significant reduction in the viral load was observed with RVR and W8EVR at week 2 compared to that at 24 h, and the SVR rates were correspondingly very high. It was believed that the reduction in the viral load at week 2 is important.

Real-time PCR assay is now commonly used and is more sensitive for detecting serum HCV than the COBAS Amplicor HCV Monitor assay Its use may have allowed viral detection for a longer period of time, possibly resulting in the number of RVR and EVR patients being reduced while the SVR rate in RVR and EVR patients was increased. Examination of the SVR rate by the timing of

HCV RNA negativity using real time PCR assay will be necessary in the future.

Reduction in the duration of treatment is being investigated for the good responders to peginterferon plus ribavirin treatment. In RVR patients who achieve HCV RNA-negativity at week 4, the SVR rate is reported to be 89% when treatment is continued for 24 weeks [11]. In this study, all patients in the RI-A (RI ≤ 1.0) and RVR area became SVR; thus they were believed to be extremely good responders. A more detailed investigation with a larger number of subjects is necessary to elucidate the question of a reduction in the duration of therapy.

The explanation of early viral kinetics by SVR, TR, and NR is highly complex and is impractical in clinical use. In this study, RI and RI-2nd calculated from early viral kinetics were used. It is believed that the simplified RI and RI-2nd are effective indices to determine the therapeutic efficacy of peginterferon therapy alone. By combining these two new indices and the indices for therapeutic efficacy of peginterferon plus ribavirin (RVR, W8EVR, W12EVR and LVR), SVR was distinguished from TR during treatment. With the aid of these indices, it is believed that a more effective peginterferon plus ribavirin treatment will be possible. We used these new indices in this study, and the measurement of HCV RNA levels was conducted using the COBAS Amplicor HCV Monitor assay. Since the range of detection of HCV is narrow with this assay, there were many patients with pretreatment HCV levels above the limit of detection. The timing of HCV RNA negativity and examination based on the HCV levels at week 1 and week 2 needs to be conducted using real time PCR assay in future studies. A larger scale study should be conducted to examine the duration of treatment for patients who are on reduced doses of peginterferon and ribavirin.

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T Cell Leukemia/Lymphoma 1 and Galectin-1 Regulate Survival/Cell Death Pathways in Human Naive and IgM+ Memory B Cells through Altering Balances in Bcl-2 Family Proteins[1]

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BCR signaling plays a critical role in purging the self-reactive repertoire, or in rendering it anergic to establish self-tolerance in the periphery. Differences in self-reactivity between human naive and IgM+ memory B cells may reflect distinct mechanisms by which BCR signaling dictates their survival and death. Here we demonstrate that BCR stimulation protected naive B cells from apoptosis with induction of prosurvival Bcl-2 family proteins, Bcl-x_L and Mcl-1, whereas it rather accelerated apoptosis of IgM+ memory B cells by inducing proapoptotic BH3-only protein Bim. We found that BCR-mediated PI3K activation induced the expression of Mcl-1, whereas it inhibited Bim expression in B cells. Phosphorylation of Akt, a downstream molecule of PI3K, was more sustained in naive than IgM+ memory B cells. Abundant expression of T cell leukemia/lymphoma 1 (Tcl1), an Akt coactivator, was found in naive B cells, and enforced expression of Tcl1 induced a high level of Mcl-1 expression, resulting in prolonged B cell survival. In contrast, Galectin-1 (Gal-1) was abundantly expressed in IgM+ memory B cells, and inhibited Akt phosphorylation, leading to Bim up-regulation. Enforced expression of Gal-1 induced accelerated apoptosis in B cells. These results suggest that a unique set of molecules, Tcl1 and Gal-1, defines distinct BCR signaling cascades, dictating survival and death of human naive and IgM+ memory B cells. *The Journal of Immunology*, 2009, 182: 1490–1499.

P rimary human peripheral B cells are made up of heterogeneous subpopulations that include a high proportion of memory B cells compared with those in rodents. Due to the advantage conferred by the usefulness of CD27 as a memory marker in humans, peripheral B cells are divided into at least three distinct subsets: naive (IgM+CD27−), IgM+ memory (IgM+CD27+), and switched memory (IgG+A+CD27+) B cells (1). Of particular interest are IgM+ memory B cells in that they do not exist in mice and could develop through the novel germinal center-independent pathways and express somatically mutated IgM Abs (2). To date, IgM+ memory B cells have been proposed to be circulating splenic marginal zone (MZ)[3] B cells and to play a critical role in the protection against encapsulated organisms (2, 3).

Although in vivo function of IgM+ memory B cells is becoming evident (4), the molecular mechanisms of activation of this subset remain poorly characterized.

Due to random rearrangements of the subunits of a functional BCR from genomic cassettes, a large proportion of developing human B cells in the bone marrow express self-reactive BCRs, but most of these potentially noxious BCRs are purged from the repertoire at several checkpoints in the bone marrow and the periphery (5). Nevertheless, up to 20% of mature naive B cells in normal peripheral blood still express low-affinity self-reactive BCRs (5). In sharp contrast, IgM+ memory B cells isolated from normal donors are devoid of such self-reactive BCRs (6). These findings suggest a distinct homeostatic control of human naive and IgM+ memory B cells.

BCR transmits the signals that are critical for both the elimination of self-reactive repertoire and the expansion of pathogen-specific repertoire. Upon BCR ligation by Ags, Lyn and Syk protein tyrosine kinases are initially activated. Syk propagates the signal by phosphorylating downstream signaling molecules. This results in activation of key signaling intermediates PI3K and phospholipase C (PLC)γ2. PI3K activates Akt kinase, which is critical for B cell survival (7). PLCγ2 activation leads to the release of intracellular Ca^{2+} and protein kinase C activation, which in turn cause activation of MAPK family kinases (ERK, JNK, and p38 MAPK) and transcription factors including NF-κB and NF-AT. These outputs subsequently connect with further-downstream molecules responsible for determining B cell fates such as survival, growth, and differentiation.

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[3] Abbreviations used in this paper: MZ, marginal zone; Gal-1, galectin-1; Tcl1, T cell leukemia/lymphoma 1; PLC, phospholipase C; h, human; EGFP, enhanced GFP; CLL, chronic lymphocytic leukemia; BAFF, B cell-activating factor of the TNF family.

The Bcl-2-regulated pathway plays a critical role in BCR-induced survival and death (8, 9). The Bcl-2 family proteins fall into three subgroups: the first subgroup including Bcl-2, Bcl-x_L, and Mcl-1 inhibits some apoptotic pathways; the second subgroup including Bax and Bak directly induces apoptosis by promoting cytochrome c release from the mitochondria; the third subgroup, called BH3-only proteins, consists of at least eight mammalian proapoptotic proteins and is activated in a stimulus-specific, as well as a cell type-specific, manner. Among Bcl-2 family proteins, a BH3-only protein Bim is particularly important in controlling lymphocyte apoptosis. Bim deficiency causes a substantial expansion of autoreactive B cells leading to autoimmune diseases (10). B cells lacking Bim are refractory to BCR-induced apoptosis (10). Bim preferentially binds anti-apoptotic Mcl-1 (11, 12). Conditional knockout of Mcl-1 causes premature death of immature and mature B cells (12), suggesting a pivotal role of Mcl-1 in B cell survival. Based on these findings, tipping the balance between Mcl-1 and Bim expression may be a critical determinant for B cell survival and death. To date, little is known about how BCR signaling dictates the survival and death of human B cell subsets via the Bcl-2-regulated pathway.

In this study, we demonstrate that BCR stimulation rescued naive B cells from apoptosis with Bcl-x_L and Mcl-1 induction, whereas it rather accelerated apoptosis of IgM$^+$ memory B cells with Bim induction. Sustained Akt activation in naive but not IgM$^+$ memory B cells appears to be critical for reciprocal expression pattern of these Bcl-2 family proteins. Moreover, we demonstrate that T cell leukemia/lymphoma 1 (Tcl1) and galectin-1 (Gal-1), abundantly expressed in naive and IgM$^+$ memory B cells, respectively, play a crucial role in regulating Akt activation, thereby affecting their survival and death via the Bcl-2-regulated pathway.

Materials and Methods

Reagents

PE-Cy5-conjugated mouse anti-human (h) CD3, -hCD4, -hCD8, -hCD11b, -hCD14, -hCD56, and -human glycophorin A mAbs; FITC-conjugated mouse anti-hCD19, -hCD69, -hCD86, -hCD95 mAbs; and PE-conjugated mouse anti-hCD27 mAb were purchased from BD Immunocytometry. FITC-conjugated goat anti-hIgM, -hIgD, -hIgG, -hIgA, rabbit anti-hGal-1 sera and recombinant hGal-1 were obtained from MBL. Goat anti-hIgM and IgG/IgA/IgM F(ab')$_2$ fragments were purchased from Jackson Immuno-Research Laboratories. Rabbit anti-human phospho-ZAP70/Syk, anti-human phospho-PLCγ2 (Y1217), anti-human phospho-JNK, anti-human phospho-ERK, anti-human phospho-Akt, anti-human Bim, anti-human Tcl1 sera, and rabbit anti-human phospho-p85/p70 S6K, anti-human phospho-NF-κB p65, and anti-human Bcl-x_L mAbs were from Cell Signaling Technology. Mouse anti-β-actin mAb and rabbit anti-human Mcl-1 sera were from Sigma-Aldrich. A PI3K inhibitor (Ly294002) was purchased from Calbiochem (EMD Biosciences).

Isolation and culture of B cell subsets

Human PBMCs were separated from buffy coats kindly provided by Fukuoka Red Cross Blood Center (Chikushino, Japan). The buffy coats originate from kind whole blood donations of RBC transfusion by healthy volunteers (age range, 18–55 years). Informed consent was obtained from all subjects. B cells were isolated with Dynabeads M450 CD19 and DETACHaBEAD CD19 (Dynal Biotech) according to the manufacturer's instructions. Isolated B cells exhibited >99.5% viability confirmed by trypan blue exclusion and >95% purity by flow cytometry. Cells were further purified by cell sorting using a FACSAria (BD Biosciences). A representative sample of human B cell subsets is shown in Fig. 1A. Cells were stained with PE-Cy5-conjugated anti-hCD3, -hCD4, -hCD8, -hCD11b, -hCD14, -hCD56, -human glycophorin A; FITC-conjugated anti-human IgG; FITC-conjugated anti-human IgA, and PE-conjugated anti-hCD27 to obtain naive (IgG$^-$A$^-$CD27$^-$), IgM$^-$ memory (IgG$^-$A$^-$CD27$^+$), and switched memory (IgG$^+$/IgA$^+$CD27$^+$) B cells. Isolated B cell subsets exhibited >95% viability confirmed by trypan blue exclusion and >99% purity by flow cytometry (Fig. 1B). Cells were cultured at 1 × 10^6 cells/ml

in a flat-bottom 96-well microtiter plate in complete RPMI 1640 supplemented with 10% FCS. Preliminary experiments showed that trace levels of phosphorylation of BCR signaling molecules are observed in B cell subsets immediately after purification probably due to mechanical stress. The cells were thus rested for a couple of hours and used for further analysis throughout the study. Consistent with a previous study (2), IgM$^+$ memory B cells exhibited a slightly higher level of IgM and a slightly lower level of IgD than did naive B cells (Fig. 1C). Absence of surface expression of CD95, CD86, and CD69, representative activation markers, in both subsets before stimulation, suggests that these cells are rested (Fig. 1C).

Expression constructs and transient transfection of human B subsets

Constructs encoding human Tcl1- or Gal-1-enhanced GFP (EGFP) fusion proteins (pEGFP-Tcl1 or -Gal-1) were generated by inserting sequence encoding the full-length protein into the pEGFP-N3 vector (Clontech). Transient transfections of B cell subsets with pEGFP-Tcl1 or pEGFP-Gal-1 were conducted using the Nucleofector protocol from AMAXA Biosystems. Cells (1 × 10^6) were suspended in 100 μl of Nucleofector solution with 5 μg of plasmid DNA and then electroporated using program U-15. Cells were transferred to 2.5 ml of medium containing 15% FCS and harvested 24 h after transfection. The transfection efficiency ranges between 20 and 30% for each experiment.

Annexin V staining

After culture, cells (1–2 × 10^5) were washed twice with PBS and then suspended in 85 μl of binding buffer (MBL) containing Ca^{2+}. Cell suspension supplemented with 10 μl of annexin V-FITC or -PE (MBL) and 5 μg of propidium iodide or 1 μg of 7-aminoactinomycin D was incubated at room temperature for 15 min in the dark. Subsequently, 600 μl of binding buffer were added, and the percentage of early apoptotic cells was measured using flow cytometry.

Mitochondria membrane potential

Assessment of mitochondria membrane potential was performed using Mitotracker Red CMXRos (Invitrogen). Cells were incubated in 50 nM Mitotracker Red at 37°C for 1 h in the dark. Flow cytometric analysis (50,000 events/sample) was performed on FACSCalibur (BD Biosciences). Cell debris was electronically gated out based on the forward scatter. Data were further analyzed using FlowJo software.

Measurement of intracellular free calcium

Cells were washed with RPMI 1640 containing 10% FCS and adjusted at 1 × 10^6 cells/ml. After incubation at 37°C for 15 min, 1 μg/ml fluo-4-acetoxymethyl esterM (Dojindo) was added, and the cells were incubated for a further 30–45 min with resuspension every 15 min. The cells were centrifuged and resuspended in RPMI 1640 at 2 × 10^6 cells/ml. The cells were stimulated with anti-IgM (20 μg/ml), and the fluorescence intensity of intracellular fluo 4 was monitored and analyzed using flow cytometry.

Western blot analysis

Unstimulated or stimulated cells (1 × 10^6) were lysed as described (13). Lysates were then denatured in an equal volume of 2× SDS sample buffer, resolved by a 10% SDS-PAGE gel and electrotransferred to nitrocellulose membranes in non-SDS-containing transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol, pH 8.5). Western blotting was performed with anti-phospho-Syk (1/2,000), anti-phospho-PLCγ2 (1/2,000), anti-phospho-p85/p70 S6 kinase (1/2,000), anti-phospho-JNK (1/2,000), anti-phospho-ERK (1/2,000), anti-phospho-Akt (1/2,000), anti-phospho-p65 NF-κB (1/2,000), anti-Bim (1/2,000), anti-Bcl-x_L (1/2,000), anti-Mcl-1 (1/5,000), anti-Tcl1 (1/2,000), anti-Gal-1 (1/2,000), and anti-β-actin (1/5,000) followed by a 1/15,000 dilution of anti-rabbit or anti-mouse HRP-conjugated IgG (Jackson ImmunoResearch Laboratories). Blots were developed with ECL plus kit (Amersham Biosciences). The chemiluminescence intensity was monitored with a laser3000 (FujiFilm) instrument. We quantitated band intensity of the proteins using ImageGauge software (FujiFilm) and normalized their expression in reference to β-actin levels. Using these normalized data, relative expression is subsequently calculated as fold changes in protein expression compared with the controls.

Quantitative real-time PCR

Total RNA was extracted from sorted human B cell subsets using Isogen reagent (Nippon Gene) and treated with DNase I (Invitrogen) to remove contaminating genomic DNA. First-strand cDNA was synthesized using a
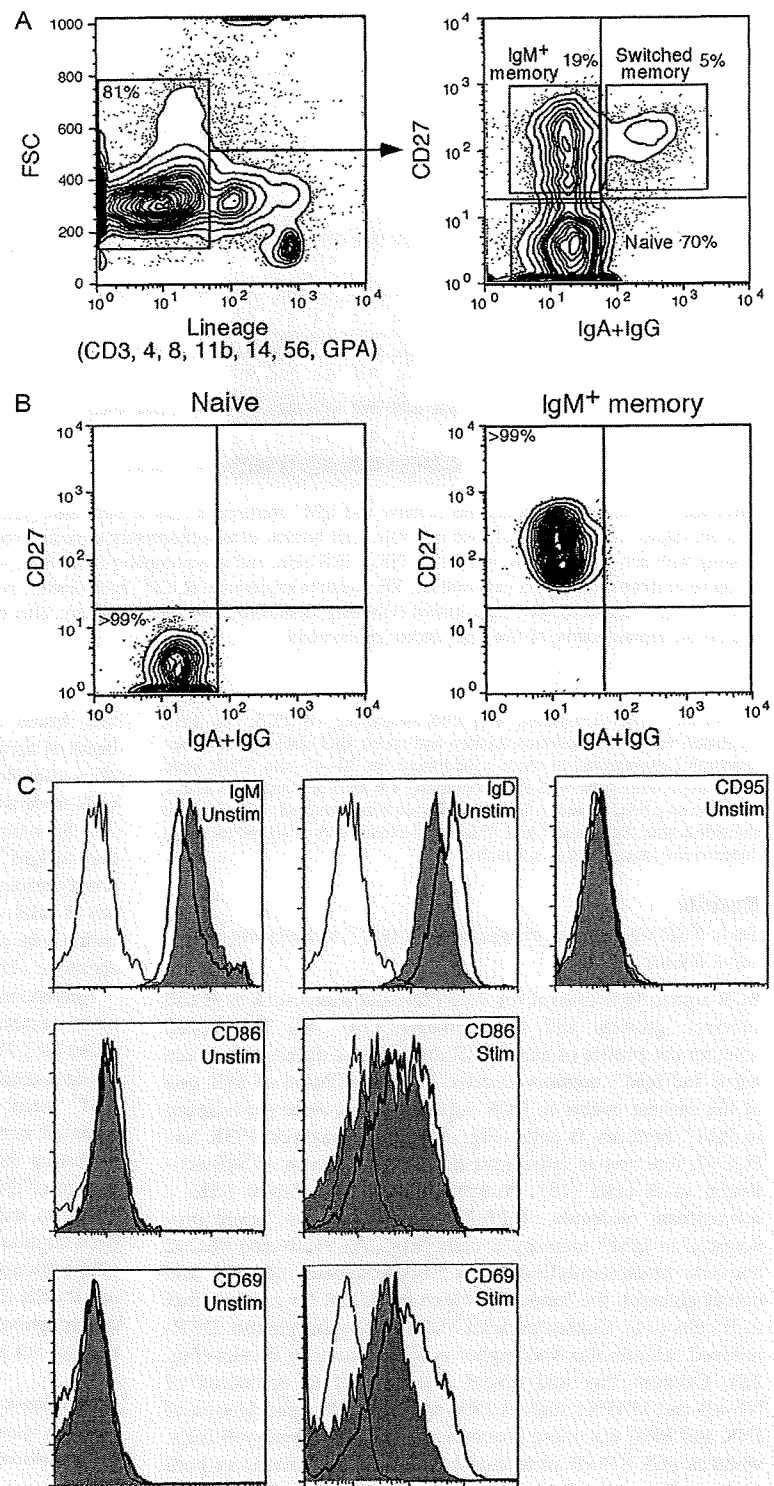
FIGURE 1. Isolation of purified human peripheral B cell subsets. *A*, Phenotypic analysis of B cell subsets in human peripheral blood. Donor B cells were purified by staining with Abs to CD3, CD4, CD8, CD11b, CD14, CD56, and glycophorin A (GPA) and were then evaluated by flow cytometry. B cell subsets were identified according to surface IgG/IgA and CD27 expression: $IgG^-A^-CD27^-$ B cells (naive), $IgG^-A^-CD27^+$ B cells (IgM^+ memory), and $IgG^+A^+CD27^+$ B cells (switched memory). Data are presented as density plots. *B*, Highly purified B cell subsets were separated after cell sorting. *C*, Surface marker expression in human B cell subsets. Purified B cell subsets before (Unstim) and after stimulation (Stim) with 20 μg/ml F(ab')$_2$ goat anti-hIgM (36 h) were analyzed separately for IgM, IgD, CD95, CD86, and CD69 surface expression. Bold line, Naive B cells; gray area, IgM^+ memory B cells; thin line, isotype control line. These results are representative of peripheral blood samples from more than 10 different donors.

QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was conducted in the ABI Prism 7700 Sequence Detector (Applied Biosystems). Reactions were performed in triplicate wells in 96-well plates. TaqMan target mixes for Bim, Bcl-x$_L$, Mcl-1, Tcl1, and Gal-1 were purchased from Applied Biosystems. 18S rRNA was separately amplified in the same plate to be used as an internal control for variances in the amount of cDNA in PCR. Collected data were analyzed with Sequence Detector software (Applied Biosystems). Data were expressed as a fold change in gene expression relative to those from unstimulated naive B cells.

Intracellular flow cytometry

After two washings with PBS containing 1% FCS, 5×10^5 cells were placed in a 96-well microtiter plate. Cells were resuspended with 50 μl of medium plus 50 μl of fixation buffer (BD Biosciences) and incubated for 10 min at 37°C. After washing again with PBS containing 1% FCS, cells were suspended with 50 μl of saponin permeabilization buffer (BD Biosciences) and spun down. The cell pellet was incubated with primary Abs (anti-human Mcl-1 or Bim) in saponin buffer at room temperature for
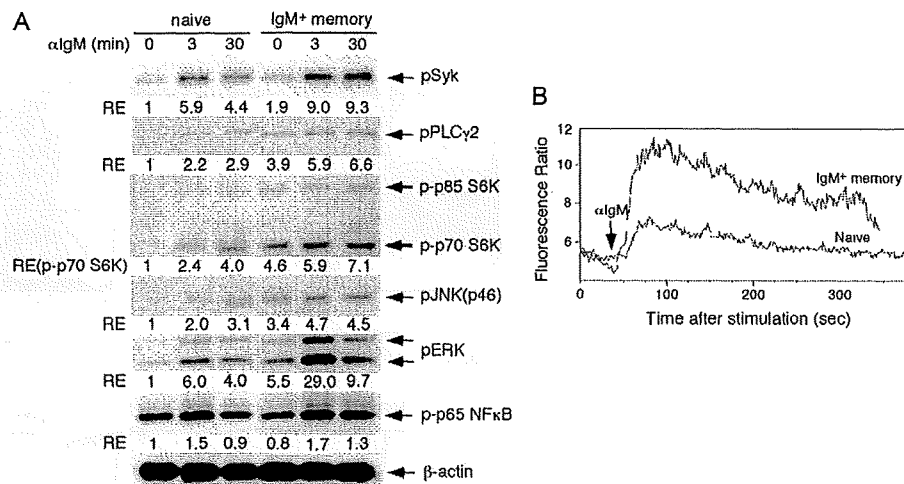
FIGURE 2. BCR signaling profiles of naive and IgM$^+$ memory B cells at early time points. *A*, B cell subsets were stimulated with 20 μg/ml F(ab')$_2$ goat anti-hIgM for the indicated time intervals. Cell lysates were subsequently separated on an 8% or 10% SDS-PAGE gel, and analyzed by Western blotting with anti-phospho-Syk, -PLCγ2, -JNK, -ERK sera, and anti-phospho-p70/85 S6K, -p65 NF-κB mAb and anti-β-actin mAb. Results are representative of three independent experiments. RE, Relative expression. *B*, Ca^{2+} mobilization in naive and IgM$^+$ memory B cells. Intracellular free calcium levels in fluo-4-acetoxymethyl ester-loaded cells were monitored using flow cytometry, after cells were stimulated with 20 μg/ml F(ab')$_2$ goat anti-hIgM. Results are representative of five independent experiments.

30–45 min. After a washing with PBS containing 1% FCS, cell were incubated with PE-conjugated donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories) at room temperature for 30–45 min. Cells were washed one more time with PBS containing 1% FCS and analyzed at low flow rate on a FACSCalibur. B cell population was identified on its forward and side scatter distribution, and 15,000 cell events were analyzed for mean fluorescence using FlowJo software.

Results

Early BCR signaling is exaggerated in IgM$^+$ memory but not naive B cells

BCR signaling is critical for B cell fate decisions such as B cell survival, growth, and differentiation (14). We first tested whether the profile of early BCR signaling is different between naive and IgM$^+$ memory B cells. Phosphorylation of Syk, one of the earliest events in BCR signaling, was more pronounced in IgM$^+$ memory B cells (Fig. 2*A*). Two enzymes, PI3K and PLCγ2, function as critical mediators downstream of Syk activation in B cells (15). Phosphorylation of p85/p70 S6K, a downstream molecule of PI3K, and PLCγ2, was more pronounced in IgM$^+$ memory B cells (Fig. 2*A*). Activated PLCγ2 converts phosphatidylinositol 4,5-bisphosphate into IP3 and diacyl glycerol, the former of which is critical for calcium flux in B cells (14). Consistent with PLCγ2 phosphorylation, BCR-induced calcium flux was higher in IgM$^+$ memory B cells (Fig. 2*B*). Calcium flux and diacyl glycerol led to activation of NF-κB and MAPKs such as JNK and ERK. Phosphorylation of JNK and ERK was more pronounced in IgM$^+$ memory B cells, whereas p65 NF-κB phosphorylation was comparable in both subsets (Fig. 2*A*). Taken together, during the early phase of BCR activation, downstream signaling is pronounced especially in IgM$^+$ memory B cells as compared with naive B cells.

BCR stimulation rescues naive but not IgM$^+$ memory B cells from apoptosis

Following anti-IgM stimulation alone, naive and IgM$^+$ memory B cells did not either divide or release Igs in the culture (data not shown), suggesting that BCR signaling alone is not sufficient to induce the growth and differentiation of human B cell subsets. We

then tested whether the BCR signaling affects the survival and death of naive and IgM$^+$ memory B cells. In the absence of stimuli, a considerable fraction of purified naive and IgM$^+$ memory B cells underwent apoptotic cell death within 2 days in vitro (Fig. 3*A*). Spontaneous cell death was more pronounced in naive B cells than in IgM$^+$ memory B cells. BCR stimulation, however, significantly rescued naive B cells from apoptosis, whereas IgM$^+$ memory B cells were not rescued (Fig. 3, *A* and *B*). Thus, BCR signaling can protect naive, but not IgM$^+$ memory B cells from apoptotic cell death.

Mitochondrial perturbations including cytochrome *c* release and inner membrane depolarization correlate with BCR-induced apoptosis (16). We thus tested whether BCR-induced depolarization of the mitochondrial inner membrane could be altered in naive and IgM$^+$ memory B cells. High levels of mitochondrial membrane potential were observed in both subsets immediately after sorting, indicating their highly viable state (Fig. 3*C*, *a* and *d*). A 2-day culture of these subsets without stimuli caused a remarkable decrease in mitochondrial membrane potential (Fig. 3*C*, *b* and *e*). BCR stimulation for 2 days, however, partially abrogated the loss of mitochondrial membrane potential in naive, but not IgM$^+$ memory B cells (Fig. 3*C*, *c* and *f*). Thus, BCR signaling rescues the B cell apoptosis pathway upstream of mitochondrial damage in naive, but not IgM$^+$ memory B cells.

BCR stimulation induces anti-apoptotic Mcl-1 in naive B cells, whereas it induces proapoptotic Bim in IgM$^+$ memory B cells at the protein level

Bcl-2 family proteins are the primary regulators of mitochondrial membrane integrity and play a vital role in the control of apoptosis (9). We tested whether BCR signaling affects gene expression of Bim, Bcl-x$_L$, and Mcl-1 in naive and IgM$^+$ memory B cells (Fig. 4*A*). Bcl-x$_L$ mRNA expression was induced after BCR stimulation in both subsets, and such induction was more pronounced in naive B cells. In contrast, the expression level of Bim mRNA was slightly higher in IgM$^+$ memory B cells irrespective of BCR stimulation. Mcl-1 mRNA expression was not significantly changed in both subsets. We next tested whether BCR signaling affects protein
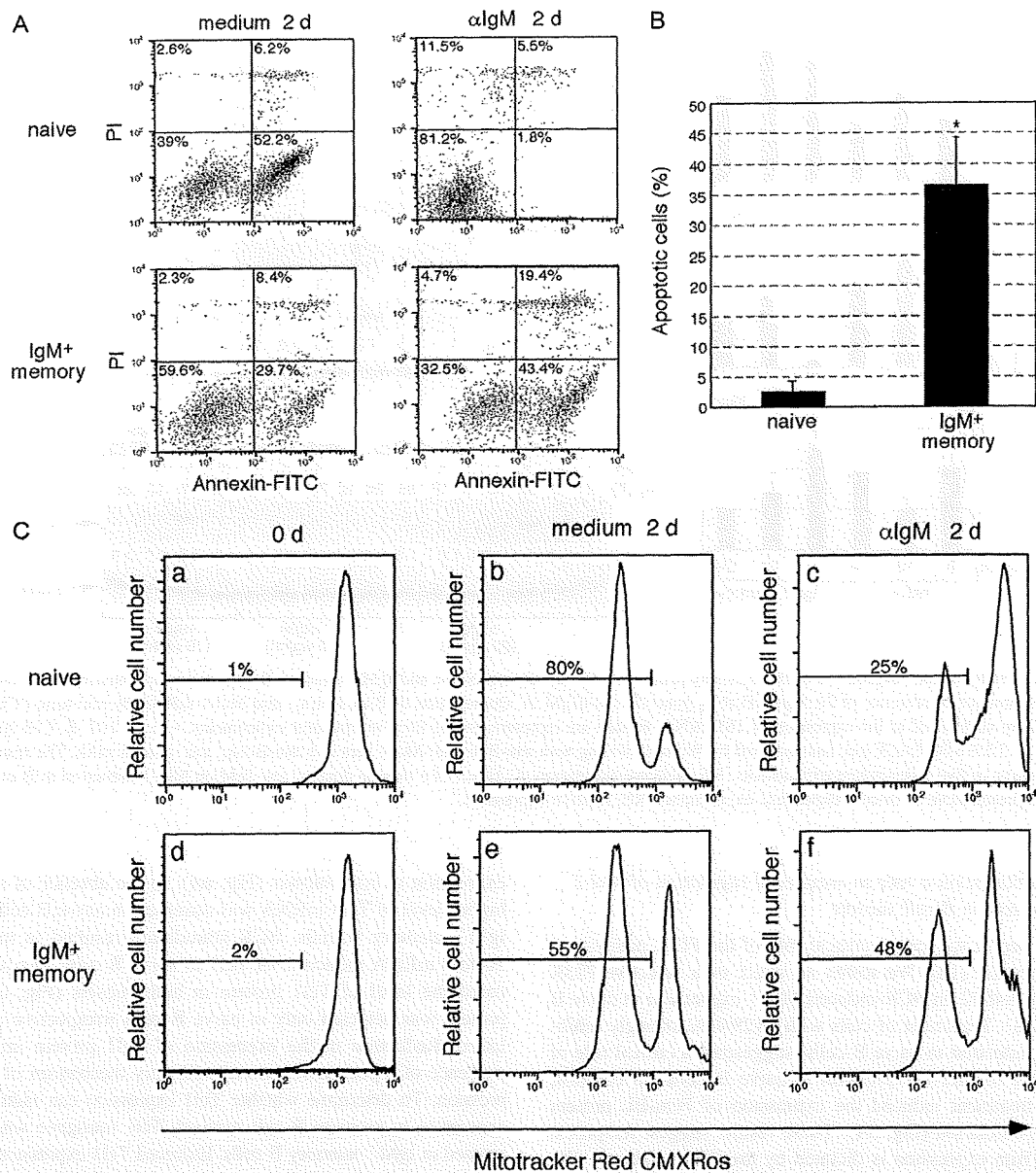
FIGURE 3. BCR-induced apoptosis in B cell subsets. *A*, Naive and IgM+ memory B cells were incubated in the absence or presence of 20 μg/ml F(ab')2 goat anti (α)-hIgM. After 2 days (2 d) of culture, cells were double-stained with FITC-labeled annexin V and propidium iodide (PI) and analyzed using flow cytometry. Apoptotic cells are visible as annexin V-positive population, both propidium iodide negative (early apoptosis) and propidium iodide positive (late apoptosis). The results shown are representative of three independent experiments. *B*, Percentage apoptosis of B cell subsets after BCR stimulation. The data are shown as the mean ± SD of six independent experiments. *, $p < 0.01$. *C*, BCR-induced mitochondrial inner membrane depolarization in B cell subsets. Naive and IgM+ memory B cells were treated for 2 days with either medium or 20 μg/ml F(ab')2 goat anti-human IgM. Percentages of Mitotracker Red CMXRos^low cells are shown. Data obtained from cells immediately after isolation are also shown. Results are representative of three independent experiments.

expression of Bim, Bcl-x_L, and Mcl-1 in naive and IgM− memory B cells (Fig. 4*B*). Three isoforms (Bim-EL, Bim-L, and Bim-S) are expressed in various cell types, including lymphocytes (17). In the absence of stimuli, Bim-EL was weakly expressed in both subsets, but in IgM+ memory B cells BCR stimulation induced all of three Bim isoforms at the level higher than those in naive B cells. On the other hand, the expression level of anti-apoptotic proteins Bcl-x_L and Mcl-1 was higher in naive B cells after BCR stimulation. Given that the difference in expression levels of surface IgM between two subsets (Fig. 1*C*) might cause these phenomena, we

tested Mcl-1 expression in naive and IgM+ memory B cells using titrated doses of anti-IgM Ab. Higher levels of Mcl-1 in naive B cells were observed at all doses of anti-IgM tested (Fig. 4*C*). Collectively, these results suggest that after BCR stimulation, anti-apoptotic Bcl-x_L and Mcl-1 are predominantly expressed in naive B cells, whereas the proapoptotic protein Bim was more abundantly expressed in IgM+ memory B cells. The discrepancy of mRNA and protein levels strongly suggests the existence of post-transcriptional regulation of Bim and Mcl-1 expression in both subsets.
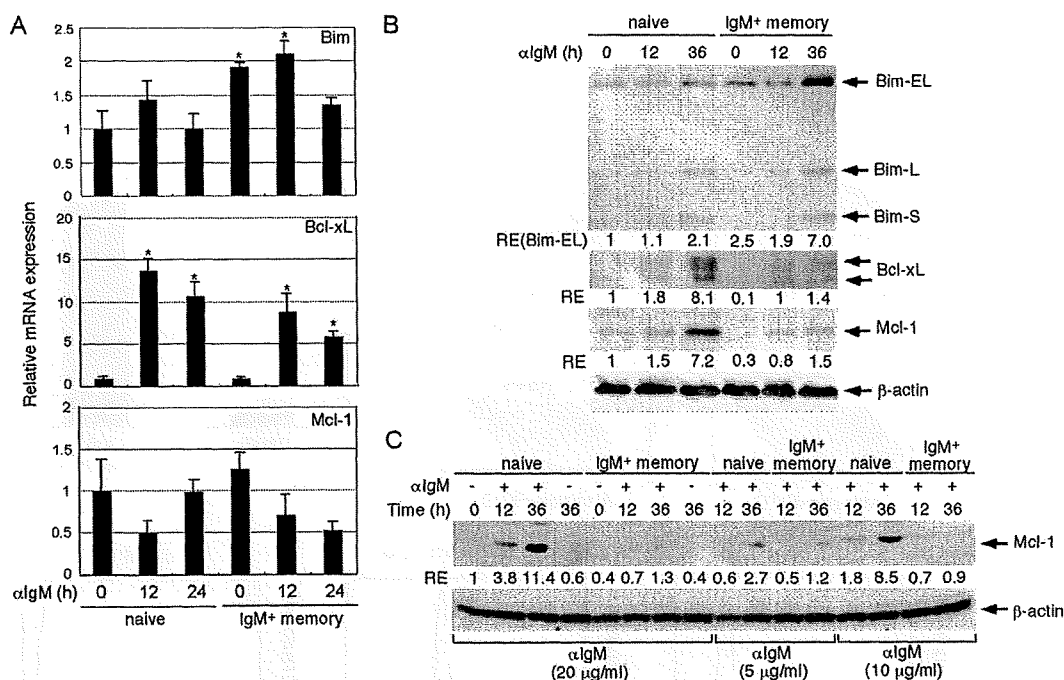
FIGURE 4. BCR-induced expression of Bcl-2 family proteins in B cell subsets. Naive and IgM⁺ memory B cells were incubated for the indicated time periods in the absence or presence of 20 mg/ml F(ab')₂ goat anti (α)-hIgM. A, Quantitation of Bim, Bcl-x_L, and Mcl-1 mRNA by real-time PCR in B cell subsets. Data are normalized to the expression of 18S rRNA. Results are representative of three independent experiments. *, $p < 0.01$. B, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Bim and -Mcl-1 sera and anti-Bcl-x_L and -β-actin mAb. The results shown are representative of five independent experiments. RE, Relative expression. C, Effect of a titrated dose of anti-IgM on Mcl-1 induction in B cell subsets. Results are representative of three independent experiments. RE, relative expression.

PI3K activation plays a role in reciprocal regulation of Mcl-1 and Bim protein in B cell subsets

A previous report suggests a critical role of the PI3K pathway in Mcl-1 expression (18). We tested an effect of a selective PI3K inhibitor Ly294002 on BCR-induced Mcl-1 expression in naive B cells (Fig. 5A). Treatment of cells with Ly294002 strongly inhibited Mcl-1 induction in naive B cells, suggesting a critical role of PI3K activity in Mcl-1 expression in naive B cells. In contrast, Ly294002 treatment induced the expression of Bim-EL protein after BCR stimulation (Fig. 5A). These results suggest reciprocal Mcl-1 and Bim expression is dictated by the PI3K pathway. We thus monitored the phosphorylation of Akt, a downstream molecule of PI3K, during BCR stimulation. Akt phosphorylation was sustained for longer periods in naive B cells than IgM⁺ memory B cells (Fig. 5, B and C). A previous study showed that PI3K activation is critical for BCR-mediated induction of CD86 and CD69 surface expression in murine B cells (19). As shown in Fig. 1C, expression levels of CD86 and CD69 are higher in naive than IgM⁺ memory B cells at a late time point. Collectively, these results suggest that reciprocal expression of Mcl-1 and Bim protein in both subsets could be explained by their distinct regulation of the PI3K pathway.

Tcl1 and Gal-1 are the critical mediators for B cell to express Mcl-1 and Bim proteins, respectively

To identify the molecule responsible for sustained activation of the PI3K pathway, we conducted gene expression profiling of B cell subsets before and after BCR stimulation. A subset of genes displayed >2-fold differences between naive and IgM⁺ memory B cells (data not shown). Among these genes, we focused on Tcl1, a potent Akt kinase coactivator (20, 21). We tested Tcl1 mRNA

expression in both subsets (Fig. 6A). In the absence of stimuli, a higher level of Tcl1 mRNA was observed in naive B cells than in IgM⁺ memory B cells. BCR stimulation resulted in more than 10-fold mRNA induction of Tcl1 in naive B cells. We next evaluated the level of Tcl1 protein in both subsets (Fig. 6B). Tcl1 protein was detected only in naive B cells irrespective of stimulation. Reduction in the expression of Tcl1 protein implies the existence of a posttranscriptional inhibitory mechanism of Tcl1 expression. To determine whether Tcl1 expression can induce Mcl-1 expression to promote B cell survival, Tcl1 transgene was overexpressed in IgM⁺ memory B cells. Enforced Tcl1 expression induced a high level of Mcl-1 expression in IgM⁺ memory B cells and protected their apoptotic cell death (Fig. 6, C and D). Thus, Tcl1 expression in naive, but not IgM⁺ memory B cells plays a critical role in Mcl-1 expression that in turn promotes their survival.

We also sought to identify a molecule involved in Bim expression and apoptosis in IgM⁺ memory B cells. In a list of genes identified in microarray analysis, higher levels of a glycoprotein Gal-1 in IgM⁺ memory B cells were noted (data not shown). In the absence of stimuli, Gal-1 mRNA was more expressed in IgM⁺ memory B cells and BCR stimulation of this subset caused drastic mRNA induction of this gene (Fig. 7A). Consistent with its mRNA expression, Gal-1 protein was abundantly expressed in IgM⁺ memory B cells (Fig. 7B). To test whether Gal-1 expression can induce Bim expression in B cells and enhance their apoptosis, Gal-1 transgene was overexpressed in naive B cells. Enforced Gal-1 expression resulted in higher levels of Bim expression in naive B cells, which is associated with the increment of apoptotic cells by >2-fold (Fig. 7, C and D). These results suggest that Gal-1 plays a vital role in promoting Bim expression and inducing apoptosis in IgM⁺ memory B cells. Interestingly, Gal-1 also functions
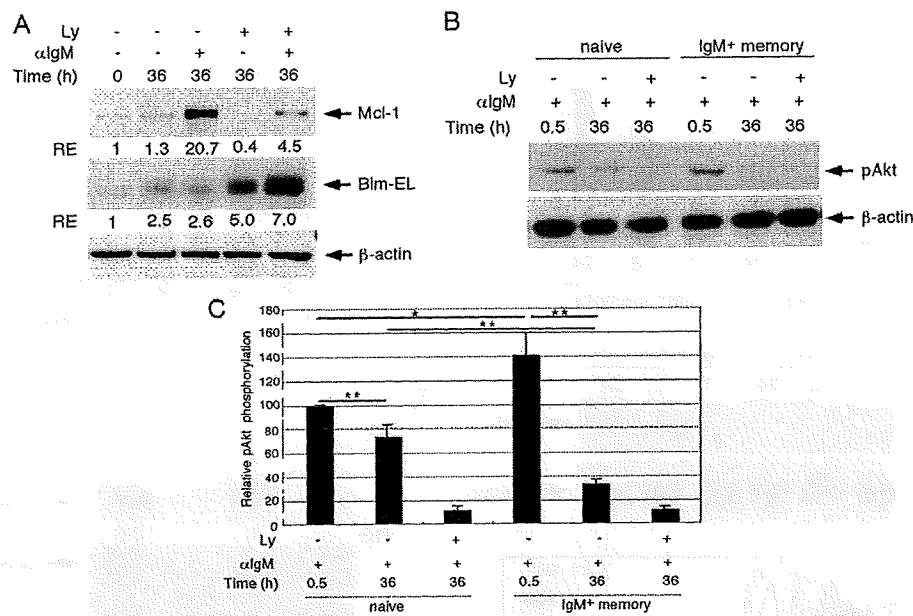
FIGURE 5. Regulation of Mcl-1 and Bim protein expression by the PI3K pathway. *A*, Naïve B cells were pretreated with or without Ly294002 (Ly; 10 μM) for 30 min and stimulated for 36 h in the absence or presence of 20 μg/ml F(ab′)₂ goat anti (α)-hIgM. Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by western blotting with anti-Bim, -Mcl-1 sera, and anti-β-actin mAb. The results shown are representative of three independent experiments. *B*, Naïve and IgM⁻ memory B cells were pretreated with or without Ly294002 (10 μM) for 30 min and stimulated for the indicated time periods in the absence or presence of 20 μg/ml F(ab′)₂ goat anti-human IgM. Cell lysates were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-phospho-Akt sera and anti-β-actin mAb. Results are representative of three independent experiments. *C*, Densitometric analyses of Akt phosphorylation in B cell subsets. The resulting values were expressed as the percentage in reference to that of BCR-stimulated naïve B cells at 0.5 h. Values are the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

as a soluble cytokine (22). Because activation of Akt and JNK is critical for regulating Bim expression (23, 24), we tested the effect of recombinant Gal-1 on BCR-induced phosphorylation of Akt and JNK in B cells. As shown in Fig. 7*E*, Gal-1 remarkably inhibited

Akt phosphorylation, whereas it slightly enhanced JNK phosphorylation in B cells upon BCR stimulation. Taken together, Gal-1 regulates Bim expression through its effects on activation of Akt and JNK in B cells.
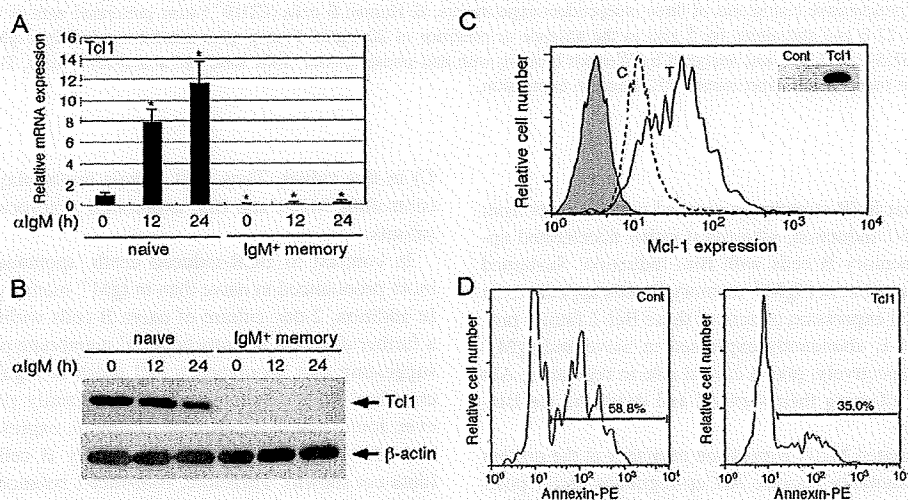

FIGURE 6. Tcl1 is critical for Mcl-1 expression and survival of B cell subsets. Naïve and IgM⁺ memory B cells were incubated for the indicated time intervals in the absence or presence of 20 μg/ml F(ab′)₂ goat anti (α)-hIgM. *A*, Quantitation of Tcl1 mRNA by real-time PCR in naïve and IgM⁺ memory B cells. Data are normalized to the expression of 18S rRNA. Results are representative of five independent experiments. *, $p < 0.01$ (with reference to unstimulated naïve B cells). *B*, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Tcl1 sera, and anti-β-actin mAb. Results are representative of four independent experiments. IgM⁺ memory B cells were transfected with either pEGFP-empty or -Tcl1 for 18 h and then stimulated with 20 μg/ml F(ab′)₂ goat anti-hIgM for 24 h. *C*, After culture, intracellular Mcl-1 expression of GFP-positive cells was analyzed by flow cytometry. *Insets*, Expression of Tcl1 transgene. Results are representative of three independent experiments. *D*, After culture, GFP-positive cells were stained with PE-labeled annexin V and analyzed using flow cytometry. Percentages of annexin-positive cells are shown. Results are representative histogram of three independent experiments. Cont, Control.
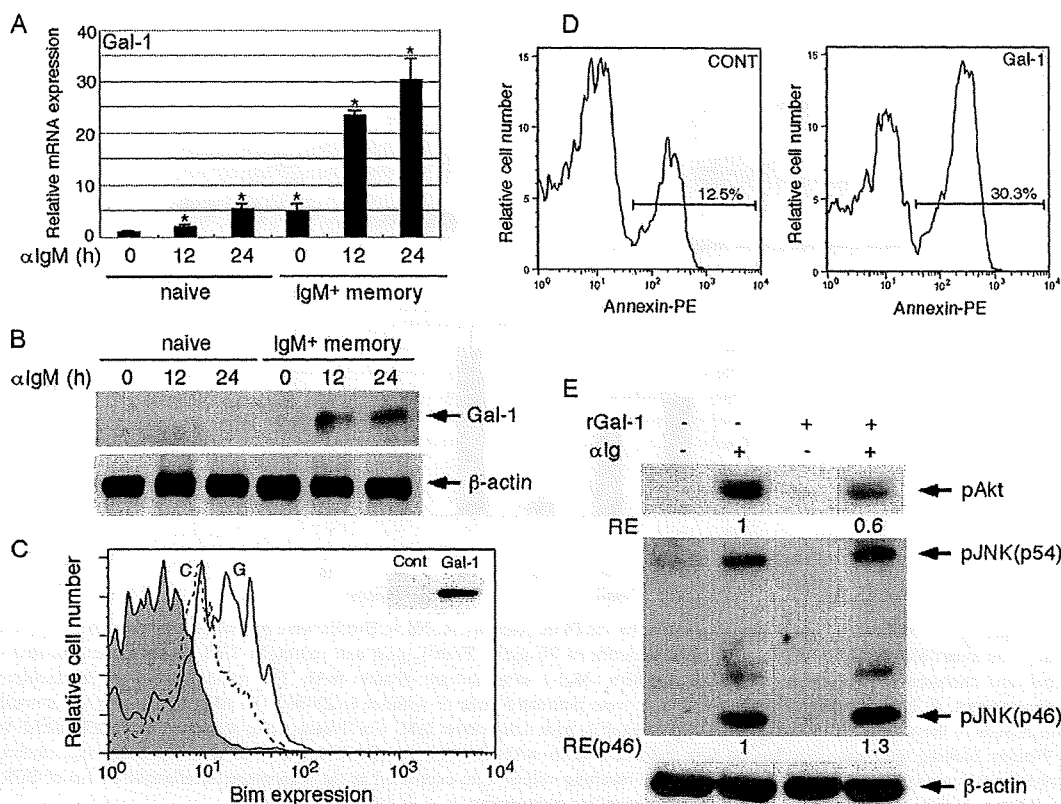
FIGURE 7. Gal-1 is critical for Bim expression and apoptosis of B cell subsets. Naive and IgM$^+$ memory B cells were incubated for the indicated time intervals in the absence or presence of 20 μg/ml F(ab')$_2$ goat anti (α)-hIgM. *A*, Quantitation of Gal-1 mRNA by real-time PCR in naive and IgM$^+$ memory B cells. Data are normalized to the expression of 18S rRNA. The results shown are representative of four independent experiments *, $p < 0.01$ (with reference to unstimulated naive B cells). *B*, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Gal-1 sera, and anti-β-actin mAb. Results are representative of three independent experiments. Naive B cells were transfected with either pEGFP-empty or -Gal-1 for 18 h and then stimulated with 20 μg/ml F(ab')$_2$ goat anti-human IgM for 24 h. *C*, After culture, intracellular Bim expression of GFP-positive cells was analyzed by flow cytometry. The insets depict the expression of *Gal-1* transgene. Results are representative of three independent experiments. Cont, Control. *D*, After culture, GFP-positive cells were stained with PE-labeled annexin V and analyzed using flow cytometry. Percentages of annexin-positive cells are shown. Results are representative histograms of three independent experiments. *E*, Human B cells (CD19$^+$) were pretreated with or without recombinant Gal-1 (10 μg/ml) for 12 h and stimulated for 5 min in the absence or presence of 20 μg/ml F(ab')$_2$ goat anti-human IgG/IgA/IgM. Cell lysates were separated on a 10% SDS-PAGE gel, and analyzed by Western blotting with anti-phospho-Akt, -JNK sera, and anti-β-actin mAb. The results shown are representative of three independent experiments. RE, Relative expression.

Discussion

Our study shows that BCR stimulation rescued naive B cells from apoptosis with Mcl-1 induction, whereas it rather accelerated apoptosis of IgM$^+$ memory B cells with Bim induction. Sustained Akt activation in naive but not IgM$^+$ memory B cells appears to be critical for reciprocal expression pattern of these Bcl-2 family proteins. Tcl1 and Gal-1, abundantly expressed in naive and IgM$^+$ memory B cells, respectively, play a crucial role in regulating Akt activation, thereby affecting their survival and death via the Bcl-2-regulated pathway.

BCR signals regulated Mcl-1 expression primarily at the protein level (Fig. 4*B*), presumably because Akt up-regulates Mcl-1 post-transcriptionally via regulating activation of glycogen synthase kinase-3 (25). Sustained Akt activation in naive B cells (Fig. 5, *B* and *C*) may thus be indispensable for continuous replenishment of Mcl-1 protein due to extraordinary short half-life of Mcl-1 (26). In contrast to Mcl-1, Bim transcription is negatively regulated by Akt through via regulating activation of the forkhead transcription factor FOXO3a (27). A small but significant increase in Bim mRNA in IgM$^+$ memory B cells (Fig. 4*A*) in response to BCR stimulation might be induced by immediate inactivation of Akt (Fig. 5, *B* and

C) in this subset. Thus, Akt signals might play a critical role in controlling Mcl-1 and Bim expression reciprocally in these B cell subsets.

In contrast to BCR-induced death, spontaneous cell death is more pronounced in naive than in IgM$^+$ memory B cells (Fig. 3*A*). In addition, 2-day culture of naive B cells without stimuli caused a further decrease in mitochondrial membrane potential (Fig. 3*C*), suggesting that spontaneous cell death is regulated at the mitochondrial level presumably by Bcl-2 family proteins. We, however, found that in the absence of stimuli, expression levels of Bim and Mcl-1 in naive and IgM$^+$ memory B cells are comparable (data not shown). Therefore, Bim-Mcl-1 balances are not the main determinant of spontaneous cell death in two subsets. Collectively, Bim-Mcl-1 balances can regulate activation-induced death of B cell subsets, whereas other Bcl-2 family proteins might be more critical for the longevity of B cell subsets in the periphery.

We show here that Tcl1 and Gal-1 are differentially expressed in human naive and IgM$^+$ memory B cells. Tcl1 interacts with Akt and functions as a potent Akt coactivator (20, 21). In Tcl1-deficient mice, the number of splenic follicular, germinal center, and MZ B cells is reduced (28). Our data suggest that Tcl1 positively

regulates Akt activation, resulting in Mcl-1 expression in B cells (Fig. 6). To date, three Tcl1 isoforms have been identified in mice and humans: Tcl1, TCL1B, and MTCP1. Our analysis showed that Tcl1 and MTCP1 but not TCL1B mRNA are expressed in human naive and IgM$^+$ memory B cells, whereas the expression level of Tcl1 mRNA is different between the subsets (data not shown). These data suggest that the difference in Tcl1 expression between the subsets (Fig. 6) does not reflect the expression patterns of Tcl1 isoforms in each subset. In contrast to Tcl1, Gal-1 induced Bim protein and enhances apoptosis in B cells (Fig. 7, *C* and *D*). Furthermore, Gal-1 significantly inhibited BCR-dependent activation of Akt, leading to the up-regulation of proapoptotic Bim (Fig. 7, *C* and *E*). Gal-1 slightly enhanced BCR-induced JNK phosphorylation (Fig. 7*E*). Because JNK activation positively regulates Bim-induced apoptosis (24, 29), Gal-1 may induce Bim expression in IgM$^+$ memory B cells also by positively regulating JNK activation.

Gal-1 may play a critical role in the maintenance of B cell tolerance. In fact, anergic B cells express higher levels of Gal-1 than wild-type cells do (30). Gal-1 induces tolerogenic dendritic cells and promotes the expansion of regulatory T cells in vivo (31). In addition, a high level of Gal-1 is required for naturally occurring CD4$^+$CD25$^+$ T cells to maintain their optimal T_{reg} function (32). These data raise an interesting possibility that human IgM$^+$ memory B cells play a critical role in the regulation of DC and Treg functions through Gal-1 production. In contrast, abnormal expression of Tcl1 could link to the pathogenesis of B cell malignancies. Tcl1-transgenic mice reveal an expansion of the CD5$^+$IgM$^+$ population that is reminiscent of human B cell chronic lymphocytic leukemia (CLL) (33), and high Tcl1 expression in human B cell CLL correlates with an aggressive CLL phenotype showing unmutated Ig variable region genes and ZAP70 positivity (34). These data collectively suggest that fine-tuning of the balance between Gal-1 and Tcl1 expression is critical for the homeostasis of human B cell subsets.

Random generation of BCRs results in the emergence of a large number of self-reactive B cells, together with pathogen-specific B cells. BCR-induced cell death and anergy are thus critical for purging or silencing self-reactive B cells. However, there are significant differences in self-reactivity between human B cell subsets: in healthy individuals: up to 20% of mature naive B cells express self-reactive BCRs, whereas IgM$^+$ memory B cells are devoid of such self-reactive BCRs (5, 6). Bim plays a critical role in BCR-induced cell death and anergy based on the fact that Bim deficiency causes a substantial expansion of autoreactive B cells leading to autoimmune diseases (10, 35). Thus, Bim expression in IgM$^+$ memory B cells may serve a novel safeguard mechanism that allows efficient elimination or inactivation of the self-reactive repertoire. Our data suggest that the balance between Mcl-1 and Bim is critical in determining B cell survival and death. It has been shown that constitutive expression of B cell-activating factor of the TNF family (BAFF), a survival-promoting cytokine for murine B cells, can break B cell tolerance through expanding self-reactive B cell populations in MZ (36, 37). BAFF exerts its effects on murine B cell survival through down-regulating Bim and up-regulating Mcl-1 (38, 39). We found that BCR-induced death in human IgM$^+$ memory B cells is abrogated in the presence of BAFF (data not shown). Because patients with systemic lupus erythematosus and Sjögren's syndrome have elevated levels of serum BAFF (37), it is important to test whether self-reactive IgM$^+$ memory B cells are expanded in these autoimmune diseases.

In summary, BCR signaling dictates survival and death in human naive and IgM$^+$ memory B cells, respectively. These phenotypes are driven by reciprocal expression of Bcl-2 family proteins

such as Mcl-1 and Bim in these B cell subsets. Tcl1 and Gal-1 are expressed in naive and IgM$^+$ memory B cell subsets, respectively. Tcl1 and Gal-1 might play critical roles in the expression of Mcl-1 and Bim, at least through regulating Akt activation. Therefore, a unique set of molecules such as Tcl1 and Gal-1 defines distinct BCR signaling cascades, dictating fate of human naive and IgM$^+$ memory B cells.

Disclosures

The authors have no financial conflict of interest.

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