

# Immunomodulatory and direct activities of ropeginterferon alfa-2b on cancer cells in mouse models of leukemia

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<b>Highlights</b>	◇ロペグインターフェロンに免疫賦活作用があること、その中でも T 細胞を活性化させていることを明らかにした。 ◇また、ロペグインターフェロンで治療されたマウスが抗腫瘍免疫記憶を獲得できていることが分かった。
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<b>Description</b>	<p>研究グループは、白血病マウスモデルを使用した実験において、ロペグインターフェロン※が生体内でがん細胞に作用するメカニズムを明らかにしました。</p> <p>ロペグインターフェロンが血液がんの治療で有望な結果が出ているとの報告が近年増加しています。しかし、そのメカニズムは明らかではありませんでした。</p> <p>本研究では、ロペグインターフェロンが腫瘍細胞に対して、直接的な細胞傷害作用をもたらしているのか、もしくは細胞そのものではなく間接的に免疫調整作用をもたらしているのかを検証しました。その結果、優れた抗腫瘍免疫作用を確認し、中でも T 細胞の活性化が重要な役割を担っていることが分かりました。これは、幅広いがん種での応用の可能性も示唆しています。加えて、白血病細胞が体内から消失したマウスに再び白血病細胞株を注入しても再発しなかったことから、免疫記憶が獲得され、再発率の低下につながる可能性があると考えられます。</p> <p>※ロペグインターフェロンは、台湾の創薬ベンチャーである PharmaEssentia Corporation が開発した新規のモノペグ化インターフェロンです。</p> <p>‘ロペグインターフェロンの作用メカニズムを解明～血液がんはじめ多種のがん治療への応用にも期待～’. 大阪公立大学. <a href="https://www.omu.ac.jp/info/research_news/entry-01073.html">https://www.omu.ac.jp/info/research_news/entry-01073.html</a> (参照 2022-06-08)</p>
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# Immunomodulatory and direct activities of ropeginterferon alfa-2b on cancer cells in mouse models of leukemia

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## Abstract

Although ropeginterferon alfa-2b has recently been clinically applied to myeloproliferative neoplasms with promising results, its antitumor mechanism has not been thoroughly investigated. Using a leukemia model developed in immunocompetent mice, we evaluated the direct cytotoxic effects and indirect effects induced by ropeginterferon alfa-2b in tumor cells. Ropiginterferon alfa-2b therapy significantly prolonged the survival of mice bearing leukemia cells and led to long-term remission in some mice. Alternatively, conventional interferon-alpha treatment slightly extended the survival and all mice died. When ropeginterferon alfa-2b was administered to interferon-alpha receptor 1-knockout mice after the development of leukemia to verify the direct effect on the tumor, the survival of these mice was slightly prolonged; nevertheless, all of them died. In vivo CD4<sup>+</sup> or CD8<sup>+</sup> T-cell depletion resulted in a significant loss of therapeutic efficacy in mice. These results indicate that the host adoptive immunostimulatory effect of ropeginterferon alfa-2b is the dominant mechanism through which tumor cells are suppressed. Moreover, mice in long-term remission did not develop leukemia, even after tumor rechallenge. Rejection of rechallenge tumors was canceled only when both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were removed in vivo, which indicates that each T-cell group functions independently in immunological memory. We show that ropeginterferon alfa-2b induces excellent antitumor immunomodulation in hosts. Our finding serves in devising therapeutic strategies with ropeginterferon alfa-2b.

## KEYWORDS

adoptive immunity, antitumor effector cells, immunomodulatory effect, interferon-alpha, ropeginterferon alfa-2b

**Abbreviations:** CTL, cytotoxic T lymphocyte; GFP, green fluorescent protein; Ifnar1, interferon-alpha receptor 1; IFN- $\alpha$ , interferon-alpha; IFN- $\gamma$ , interferon-gamma; JAK-Stat, Janus kinase-signal transducer and activator of transcription; MFI, median fluorescence intensity; PEG-IFN, pegylated interferon-alpha; p-STAT1, phosphorylation of signal transducer and activation of transcription 1; rIFN- $\alpha$ , recombinant mouse interferon-alpha; ropeg, ropeginterferon alfa-2b; STAT1, signal transducer and activation of transcription 1; T<sub>CM</sub>, central memory T; TCR, T-cell receptor; T<sub>EM</sub>, effector memory T; T<sub>N</sub>, naïve T; T<sub>SCM</sub>, stem cell-like memory T.

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## 1 | INTRODUCTION

Interferon-alpha is a pleiotropic cytokine that was first identified as a viral replication inhibitor. Nevertheless, the IFN- $\alpha$  function has since been extended to cancer suppression.<sup>1-3</sup> IFN- $\alpha$  is now approved for the treatment of both solid and hematologic tumors.<sup>4-8</sup> The underlying mechanisms of the IFN- $\alpha$  function in tumor suppression are currently a widely studied subject, and it has long been thought that IFN- $\alpha$  suppresses tumor development through its direct functions in tumor cells.<sup>9,10</sup> Also, IFN- $\alpha$  may attenuate tumor progression by activating host immune cells.<sup>11-14</sup> Despite these findings, it remains unclear whether IFN- $\alpha$  exerts its antitumor effects through stimulation of the host immune system, by a direct effect on tumor cells, or both.<sup>15</sup>

Ropeginterferon alfa-2b (ropeg) is a monopegylated IFN- $\alpha$  that was developed for the treatment of myeloproliferative neoplasms. Contrary to other PEG-IFN compounds, ropeg comprises a single positional isomer resulting in an extended elimination half-life, enabling less frequent dosing (every other week or monthly during maintenance therapy), and shows promising results for the treatment of patients with polycythemia vera.<sup>16-18</sup> Despite increasing clinical evidence, the mechanism by which ropeg treatment controls tumor cells remains unclear. In this study, we used an immunocompetent mouse model mimicking acute leukemia and showed its antitumor mechanism from both the direct and indirect effects of ropeg.

## 2 | MATERIALS AND METHODS

### 2.1 | Ex vivo and in vivo experiments

Mice, leukemia cell lines, flow cytometry analysis, next-generation sequencing analysis, drug reagents, and study design are described in Appendix S1. Animal experiments were approved by Osaka City University Animal Ethics Committee and performed according to the institutional guidelines of Osaka City University.

### 2.2 | Statistical analysis

*P* values were calculated using a two-tailed Student's *t* test, nonparametric Mann-Whitney *U* test, or Kruskal-Wallis test. The log-rank test was used for the analysis of survival. *P* values of <0.05 were considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing).<sup>19</sup>

## 3 | RESULTS

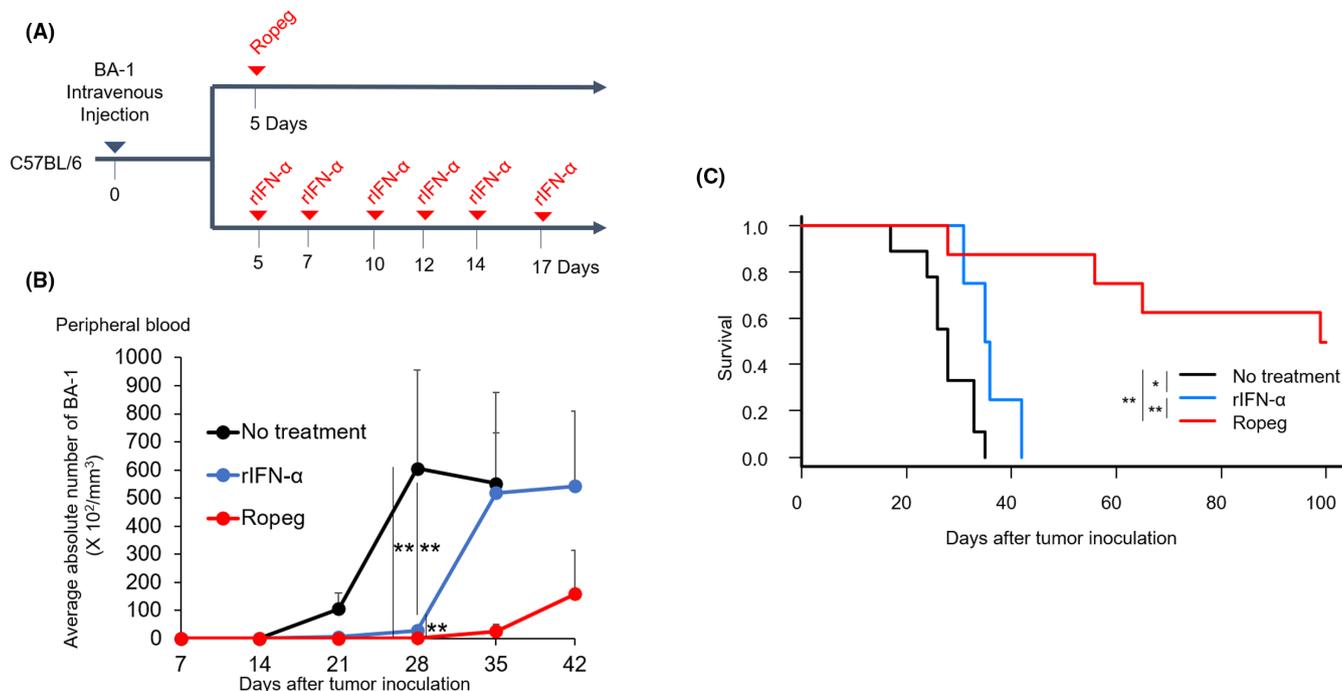
### 3.1 | Ropeginterferon alfa-2b decreases the leukemia burden in vivo and leads to the long-term survival of mice

To assess the in vivo efficacy of ropeg for leukemia, we utilized BA-1, a murine B-cell leukemia cell line that shows aggressive development, leading to death with infiltration of the bone marrow, blood, and spleen after injection into normal C57BL/6 mice without prior treatment (data not shown). To investigate the overall therapeutic potential of ropeg treatment regarding leukemia, we inoculated C57BL/6 mice with BA-1 cells via tail vein injection (Figure 1A). A significant reduction in the tumor burden in the peripheral blood was observed, especially in the ropeg-treated group (Figure 1B). We next evaluated the effects of ropeg treatment on the survival of the mice. Ropeg treatment led to long-term remission in four of eight (50%) mice, whereas all mice in the rIFN- $\alpha$  treatment group died at approximately 40 days, which demonstrated the potential of stronger antitumor effect of ropeg in vivo in comparison with rIFN- $\alpha$  in leukemia treatment. Increasing the dose of rIFN- $\alpha$  did not contribute to prolonged survival (Figure S1). The antitumor effect of ropeg was also tested in a tumor model using EL4, a murine T-cell leukemia cell line (Figure S2A). Ropeg treatment significantly delayed tumor growth of EL4 tumors, whereas rIFN- $\alpha$  treatment did not prevent tumor growth (Figure S2B).

### 3.2 | Ropeginterferon alfa-2b increases apoptosis and cell cycle arrest of leukemic cells in a time-dependent manner

We intended to evaluate the direct cytotoxic effect of ropeg and rIFN- $\alpha$  on the proliferation of BA-1 cells in vitro to elucidate the mechanism of leukemia rejection. Both ropeg and rIFN- $\alpha$  suppressed BA-1 growth, which was more pronounced in the ropeg-treated group (Figure 2A). The same result was observed in EL4 (Figure S3).

The apoptosis of BA-1 cells was significantly increased after exposure to ropeg and rIFN- $\alpha$ . In terms of total cells, the ropeg group showed an increase in early- and late-apoptotic cells in a time-dependent manner, whereas the ratio of early and late apoptotic cells was most remarkable in the rIFN- $\alpha$  group after 24 hours (Figure 2B). Ropeg significantly increased early and late apoptotic cells in comparison with rIFN- $\alpha$  and control 72 hours after the start of coculture (Table 1). Regarding viable cells, ropeg induced an increase in the MFI of PE-annexin over time, whereas rIFN- $\alpha$  peaked at 24 hours



**FIGURE 1** Ropeg treatment in BA-1 cell-injected mice. A, A schematic illustration of the therapy protocol for in vivo experiments. B, Single-cell suspensions of blood at 7, 14, 21, 28, 35, and 42 d after tumor inoculation were analyzed via flow cytometry to determine the fraction of GFP+ cells (BA-1 cells). Error bar indicates the mean and SE. Statistical significance was determined using Student's *t* test with comparisons indicated by brackets (\*\**p* < 0.01). C, Survival data in C57BL/6 mice were plotted in a Kaplan-Meier survival curve, and statistical significance was calculated with the log-rank test. No treatment (*n* = 11), rIFN- $\alpha$ -treated (*n* = 8), and ropeg-treated C57BL/6 (*n* = 8) mice. Data were derived from at least three independent experiments (\**p* < 0.05; \*\**p* < 0.01)

and did not increase thereafter (Figure 2C). IFN- $\alpha$  induces apoptosis in malignant cells via activation of caspase-3.<sup>20,21</sup> We confirmed that ropeg promoted a time-dependent increase in caspase-3 expression, which was not observed with rIFN- $\alpha$  (Figure 2D). Additionally, cell cycle analyses were performed under the same culture conditions described in the apoptosis assay. When BA-1 cells were cocultured with ropeg or rIFN- $\alpha$ , the G1 phase of the cells increased (Figure 2E). The increase in the G1 phase after 72 hours was more remarkable in the ropeg group (Table 1). IFN- $\alpha$  has been shown to induce p21, which inhibits the active cyclin D/CDK4 complex, resulting in G1 phase arrest.<sup>22-24</sup> We found that exposure to rIFN- $\alpha$  induces an increase in p21 expression in BA-1 cells, which peaks out at 24 hours, whereas exposure to ropeg induces an increase in its expression over time (Figure 2F).

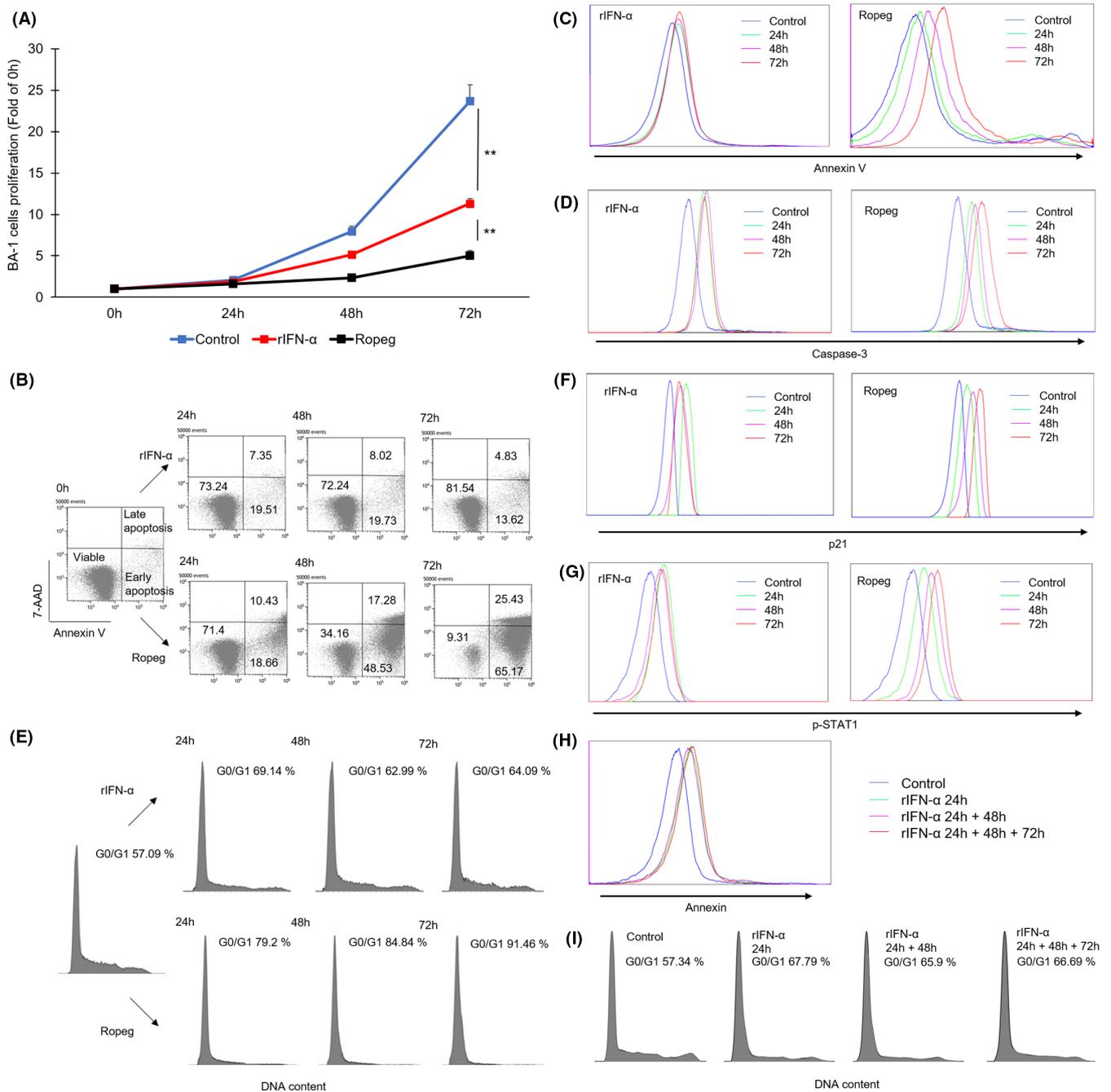
When type I IFN binds to a receptor consisting of Ifnar, STAT1 undergoes tyrosine phosphorylation by the JAK-Stat pathway. Thus, we checked whether ropeg promoted p-STAT1. The MFI of p-STAT1 was increased via coculture with ropeg or rIFN- $\alpha$ . Additionally, the MFI of p-STAT1 increased in a time-dependent manner in the ropeg-treated group, which was in line with the time-dependent effects observed in the apoptosis and cell cycle assays (Figure 2G). We administered rIFN- $\alpha$  continuously every 24 hours in the medium culturing BA-1. Annexin expression and G1 phase ratio of the BA-1 cells peaked at 24 hours after the start of culture and did not increase over time thereafter (Figure 2H,I).

### 3.3 | Loss of indirect effect attenuates the antileukemic effect of ropeg in vivo

Based on the direct cytotoxic effects of ropeg for BA-1 cells confirmed in vitro, we next investigated the direct effect on leukemia cells in vivo (Figure 3A). Both ropeg and rIFN- $\alpha$  therapies significantly enhanced the survival of *Ifnar1*<sup>-/-</sup> mice bearing BA-1 cells, in which these therapies do not act systemically. Ropeg treatment led to a survival benefit in comparison with rIFN- $\alpha$  treatment. Although ropeg treatment led to the long-term remission of leukemia in some immunocompetent mice, all *Ifnar1*<sup>-/-</sup> mice died within 17-24 days after tumor inoculation (Figure 3B). The direct action of ropeg on EL4 cells did not inhibit tumor cell growth in *Ifnar1*<sup>-/-</sup> mice (Figure S4A,B). These findings demonstrate that the immune system plays a prominent role in the ropeg-mediated antitumor effect in vivo.

### 3.4 | Indirect antitumor effects of ropeg significantly inhibit leukemia cell growth in vivo and lead to the long-term survival of mice

Based on these findings, we next investigated whether the tumor-suppressive effects and prolonged survival of leukemia-bearing mice, which we observed in ropeg-treated wild-type mice, could be



**FIGURE 2** Direct effect of ropeg and rIFN- $\alpha$  on BA-1 cells ex vivo. This section describes the drug-induced antitumor proliferative effects of coculturing BA-1 cells with ropeg (5  $\mu$ g/ml in media) and rIFN- $\alpha$  (1250  $\mu$ g/ml in media) for 72 h. A, Tumor growth curves of BA-1. Error bar indicates the SE. Statistical significance was determined using Student's *t* test with comparisons indicated by brackets (\*\**p* < 0.01). Data were derived from three independent experiments. B, Graphical representation of annexin V and 7-AAD expression of BA-1 cells after incubation with ropeg and rIFN- $\alpha$ . Representative histogram plots representing the expression of (C) annexin V, (D) caspase-3, (F) p21, and (G) p-STAT1 in viable BA-1 cells after incubation with ropeg and rIFN- $\alpha$ . (E) Representative DNA histogram plots of the cell cycle profile of BA-1 cells after incubation with ropeg and rIFN- $\alpha$ . G0/G1 percentages show representative data obtained from three experiments. (H) Annexin V expression in viable BA-1 after additional administration of rIFN- $\alpha$  (1250 U/ml every 24 h). (I) Histogram plots representing cell cycle in BA-1 cells after an additional dose of rIFN- $\alpha$  (1250 U/ml every 24 h). Histogram and dot plots in this section are representative based on three independent and reproducible experiments

confirmed based on indirect effect alone. To confirm this, we generated BA-1/*Ifnar1*<sup>-/-</sup> by deleting the *Ifnar1* gene using the CRISPR/Cas9 system, on which ropeg did not have a direct antitumor effect. (Figure 4A,B). The indirect antitumor effect of ropeg was evaluated by inducing BA-1/*Ifnar1*<sup>-/-</sup> leukemia in wild-type C57BL/6 mice

(Figure 4C). Ropeg treatment significantly prolonged the survival of mice in comparison with the rIFN- $\alpha$ -treated and untreated groups (Figure 4D). Furthermore, ropeg treatment introduced long-term remission in four of the six (67%) treated mice, which was confirmed in ropeg-treated mice with normal BA-1. These results showed that

TABLE 1 Apoptosis and cell cycle phase distribution of BA-1 cells after coculture with rIFN- $\alpha$  and ropeg

	Apoptosis distribution (%)			Cell cycle phase (%)		
	Viable cells	Early apoptosis	Late apoptosis	G0/G1	S	G2/M
Control	81.3 $\pm$ 2.0	12.0 $\pm$ 3.7	6.7 $\pm$ 2.9	56.1 $\pm$ 0.5	25.2 $\pm$ 0.6	10.3 $\pm$ 0.4
rIFN- $\alpha$ 1250 U/ml	71.5 $\pm$ 3.7	12.3 $\pm$ 1.9	15.3 $\pm$ 4.0	66.0 $\pm$ 2.0 <sup>a</sup>	26.1 $\pm$ 1.8 <sup>a</sup>	7.9 $\pm$ 0.3 <sup>a</sup>
Ropeg 5 $\mu$ g/ml	31.1 $\pm$ 11.1 <sup>a,b</sup>	31.5 $\pm$ 18.8	36.6 $\pm$ 5.9 <sup>a,b</sup>	86.1 $\pm$ 2.7 <sup>a,b</sup>	10.9 $\pm$ 1.9 <sup>a,b</sup>	3.0 $\pm$ 0.9 <sup>a,b</sup>

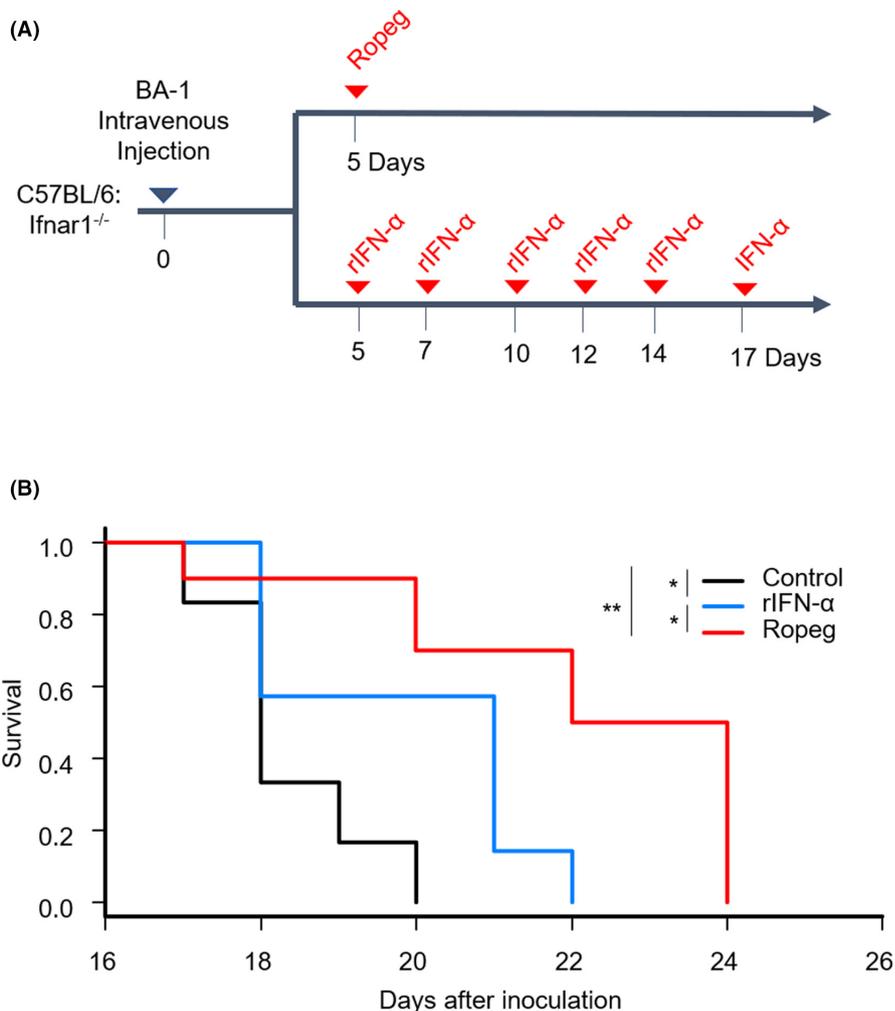
Note: BA-1 cells were exposed to compounds at the indicated concentrations for 72 h before analysis was performed. The data presented correspond to the mean  $\pm$  standard error of the mean of three independent experiments. All experiments were performed at least three times with similar results. Abbreviations: rIFN- $\alpha$ , recombinant interferon-alpha; ropeg, ropeginterferon alfa-2b.

<sup>a</sup> $p < 0.05$  compared with the control group.

<sup>b</sup> $p < 0.05$  compared with the rIFN- $\alpha$  group.

FIGURE 3 Direct antiproliferative effect of ropeg for BA-1 cells in vivo.

A, BA-1 cells ( $1 \times 10^5$ ) were injected intravenously into *lfnar1*<sup>-/-</sup> C57BL/6 mice. B, Survival data of *lfnar1*<sup>-/-</sup> C57BL/6 mice were plotted in a Kaplan-Meier survival curve, and statistical significance was calculated with the log-rank test. No treatment ( $n = 6$ ), rIFN- $\alpha$ -treated ( $n = 7$ ), and ropeg-treated C57BL/6 ( $n = 10$ ) mice. ( $*p < 0.05$ ) Data were derived from three independent experiments



under an immunocompetent condition, the dominant factor in the prolongation of survival in ropeg-treated mice is an indirect effect, such as enhancing antitumor immune response.

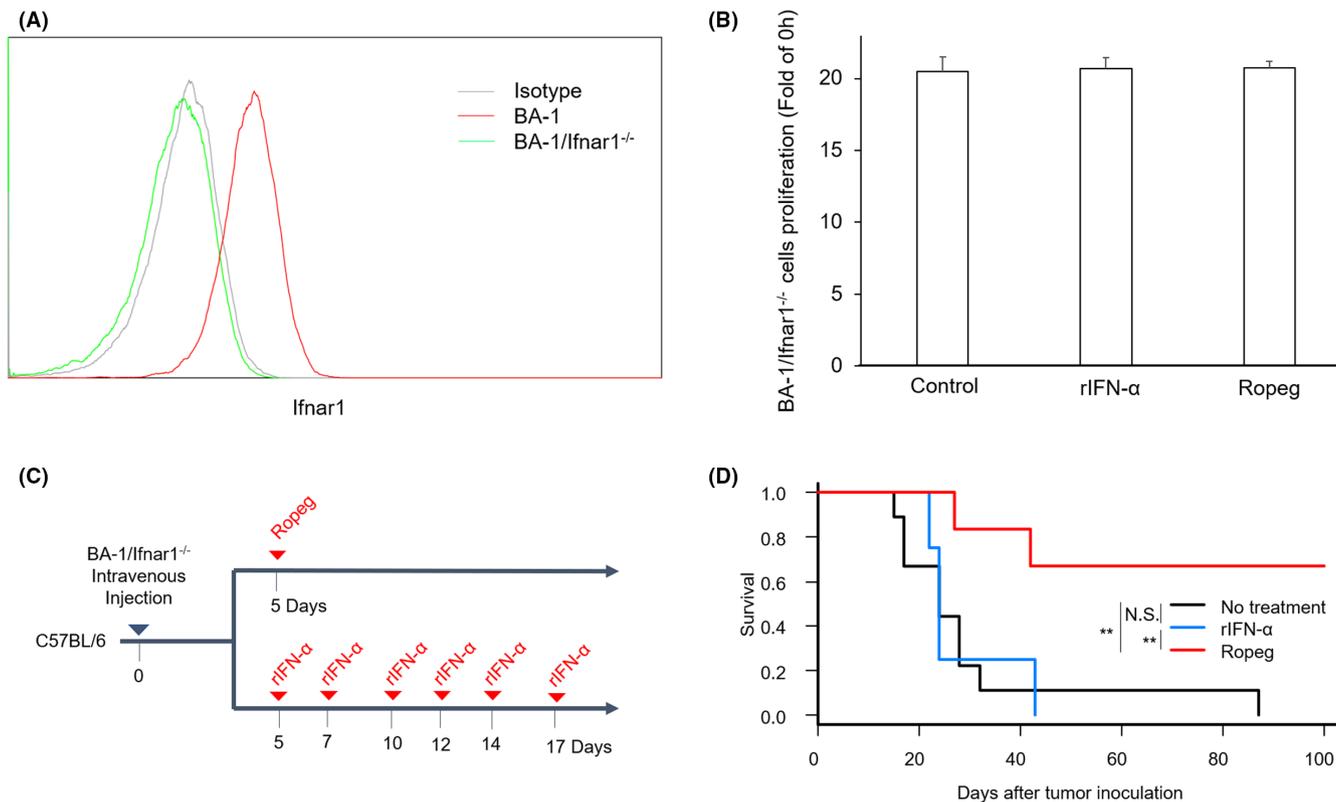
### 3.5 | Changes in splenic lymphocytes after the administration of ropeg

These results suggested that ropeg treatment exerts a more critical antitumor effect than the conventional IFN- $\alpha$  due to the immunomodulation in mice. Thus, we investigated the proportion and the

absolute number of splenic lymphocytes after the administration of ropeg or rIFN- $\alpha$  in comparison with drug-free mice.

As shown in Figure 5A, we characterized the lymphocyte subsets after the administration of ropeg or rIFN- $\alpha$ . The spleen weights and the absolute number of splenocytes in the ropeg-injected group were significantly increased in comparison with the control and rIFN- $\alpha$  groups (Figure 5B).

Ropeg-treated mice displayed increased proportions and absolute numbers of CD19<sup>+</sup> B cells and CD3<sup>+</sup> T cells in comparison with the control and rIFN- $\alpha$  groups (Figure 5C,D). CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be further categorized into memory and naïve phenotypes



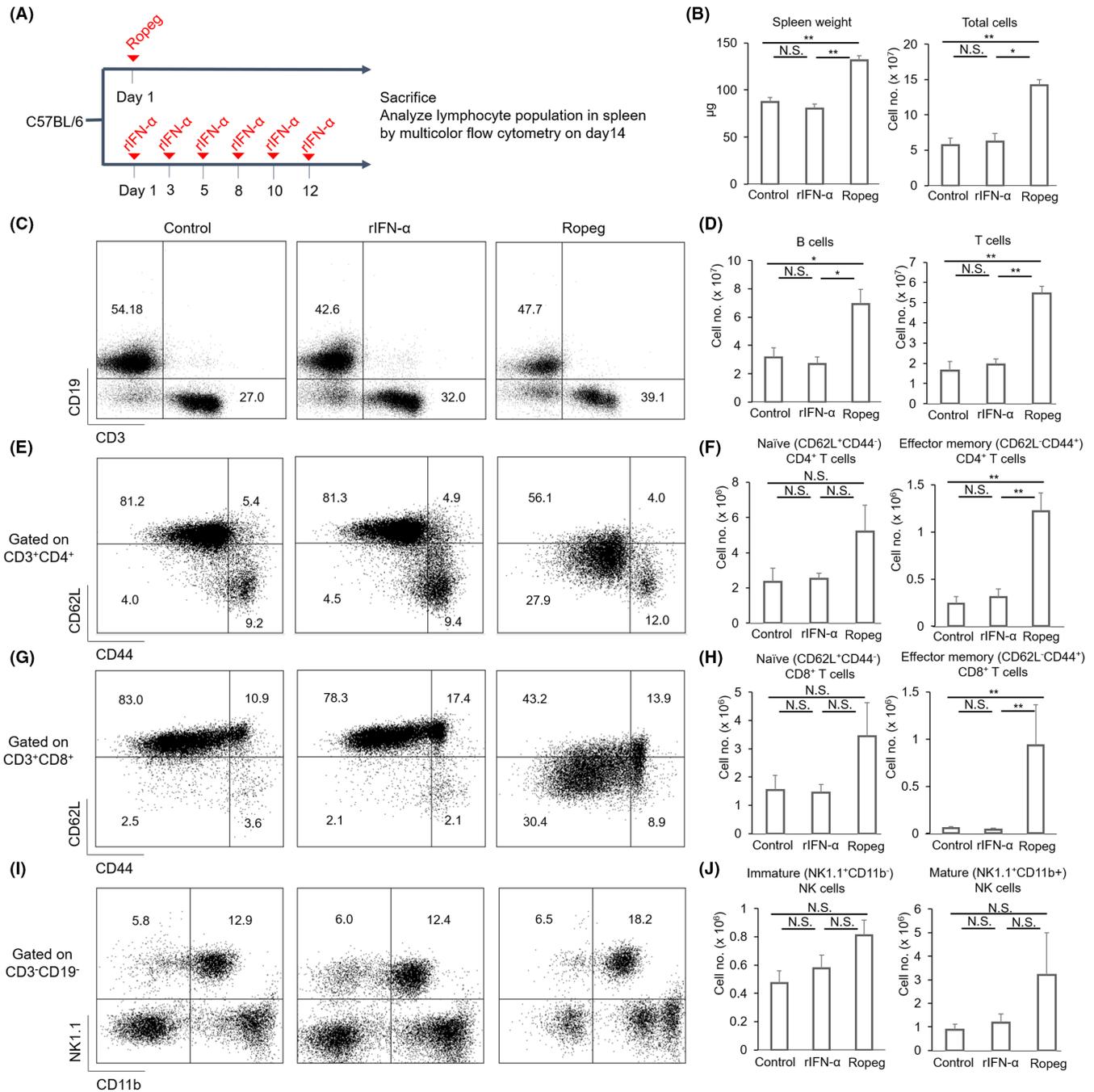
**FIGURE 4** Indirect antiproliferative effect of ropeg on BA-1 cells in vivo. A, Histogramical representation of Ifnar1 in BA-1 and BA-1/Ifnar1<sup>-/-</sup> cells. Histograms showed several cells per channel (vertical axis) versus Ifnar1<sup>-/-</sup> (horizontal axis). B, Tumor growth of BA-1/Ifnar1<sup>-/-</sup> at 72 h after incubation with ropeg (5 μg/ml in media) and rIFN-α (1250 U/ml in media). Data are derived from three independent experiments ( $n = 9$  per group). Bar charts indicate the mean and SE. C, BA-1/Ifnar1<sup>-/-</sup> cells ( $1 \times 10^5$ ) were injected intravenously into C57BL/6 mice. D, Survival data in C57BL/6 mice were plotted in a Kaplan-Meier survival curve, and statistical significance was calculated with the log-rank test. No treatment ( $n = 9$ ), rIFN-α-treated ( $n = 4$ ), and ropeg-treated C57BL/6 ( $n = 6$ ) mice. (\*\* $p < 0.01$ ) Data were derived from three independent experiments. N.S., not significant

based on the CD62L (L-selectin) and CD44 expression, with the CD44<sup>-</sup>CD62L<sup>+</sup> population considered T<sub>N</sub> cells, CD44<sup>+</sup>CD62L<sup>+</sup> population considered T<sub>CM</sub> cells, and the CD44<sup>+</sup>CD62L<sup>-</sup> population considered effector and T<sub>EM</sub> cells. As shown in Figure 5E,G, we observed the percentage of CD3<sup>+</sup> T cells with a striking downregulation of CD62L in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations in the ropeg-treated group in comparison with the control and rIFN-α groups. The numbers of T<sub>EM</sub> cells in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the ropeg-treated mice significantly increased in comparison with the rIFN-α-treated and control groups, whereas the numbers of T<sub>N</sub> cells were equivalent in each of the groups (Figure 5F,H). Ropeg significantly increased the absolute number of CD4<sup>+</sup> T<sub>CM</sub> cells compared with the control group, whereas it was comparable between the ropeg and rIFN-α groups. There was no significant difference in the absolute number of CD8<sup>+</sup> T<sub>CM</sub> cells between the ropeg group and the control or rIFN-α groups (data not shown). Recently, T<sub>SCM</sub> cells have been reported as a new immune biomarker to assess long-term memory T-cell immune reconstitution.<sup>25,26</sup> T<sub>SCM</sub> cells have been shown to differentiate into T<sub>CM</sub> and T<sub>EM</sub>.<sup>27</sup> In mice, this population is identified as an Sca-1<sup>+</sup>CXCR3<sup>+</sup> subset in T<sub>N</sub>-appearing CD44<sup>-</sup>CD62L<sup>+</sup> cells.<sup>28,29</sup> We identified an increased proportion of T<sub>SCM</sub> in the T<sub>N</sub> fraction in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells after ropeg administration

(Figure S5A,B). We also found that the larger percentage of both CD4<sup>+</sup> and CD8<sup>+</sup> cells after ropeg treatment expressed cytotoxic cytokines such as IFN-γ and perforin (Figure S6). To examine the clonality of T cells after ropeg administration, TCRβ repertoires in T cells isolated from spleens were analyzed using next-generation RNA-sequencing technology. T cells in mice after ropeg administration showed a polyclonal TCR repertoire as well as in control mice (Figure S7A,B). The Shannon-Weaver index  $H'$  of the control group and that of the ropeg-treated group were similar, indicating that T cells expanded polyclonally and maintained their diversity after ropeg treatment.

Natural killer (NK) cell maturation is a process by which lineage-committed NK cells acquire their full effector functions.<sup>30</sup> The low and high expression of CD11b divides NK cells into immature and mature subsets, respectively.<sup>31</sup> The numbers of both immature (NK1.1<sup>+</sup>CD11b<sup>-</sup>) and mature (NK1.1<sup>+</sup>CD11b<sup>+</sup>) NK cells tended to increase in the ropeg-treated group. Nevertheless, there were no significant differences in each group (Figure 5I,J).

Hence, in mice, ropeg treatment increased B cells, T cells, and NK cells in the spleen. Moreover, the dominant subsets of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were changed from T<sub>N</sub> cells to T<sub>EM</sub> cells in ropeg-treated mice.



**FIGURE 5** Changes in the percentage and absolute number of lymphocyte subsets in the spleen after the administration of ropeg. (A) A schematic illustration of the therapy method in the in vivo experiments. (B) Spleen weights and the total number of splenocytes. Splenocytes were analyzed for the expression of (C) CD3 and CD19 gated on available cells, CD62L and CD44 gated on each (E) CD3<sup>+</sup>CD4<sup>+</sup> and (G) CD8<sup>+</sup> T cells, and (I) NK1.1 and CD11b gated on CD3<sup>+</sup>CD19<sup>-</sup> cells. Representative dot plots in each group. The number in each quadrant shows the mean percentage of each population. The total number of (D) B (CD19<sup>+</sup>) cells, T (CD3<sup>+</sup>) cells, naïve (CD62L<sup>+</sup>CD44<sup>-</sup>) and effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>) cells in (F) CD4<sup>+</sup> and (H) CD8<sup>+</sup> T cells, and (J) immature and mature NK cells in each treatment group. Results are representative of three independent experiments with five mice in each group. Statistical analyses were performed using a nonparametric Mann-Whitney *U* test (\**p* < 0.05; \*\**p* < 0.01)

### 3.6 | Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells eliminates the antitumor effect of ropeg in vivo

Based on these findings, we then aimed to identify which immune cell types were crucial for the survival benefit that we observed

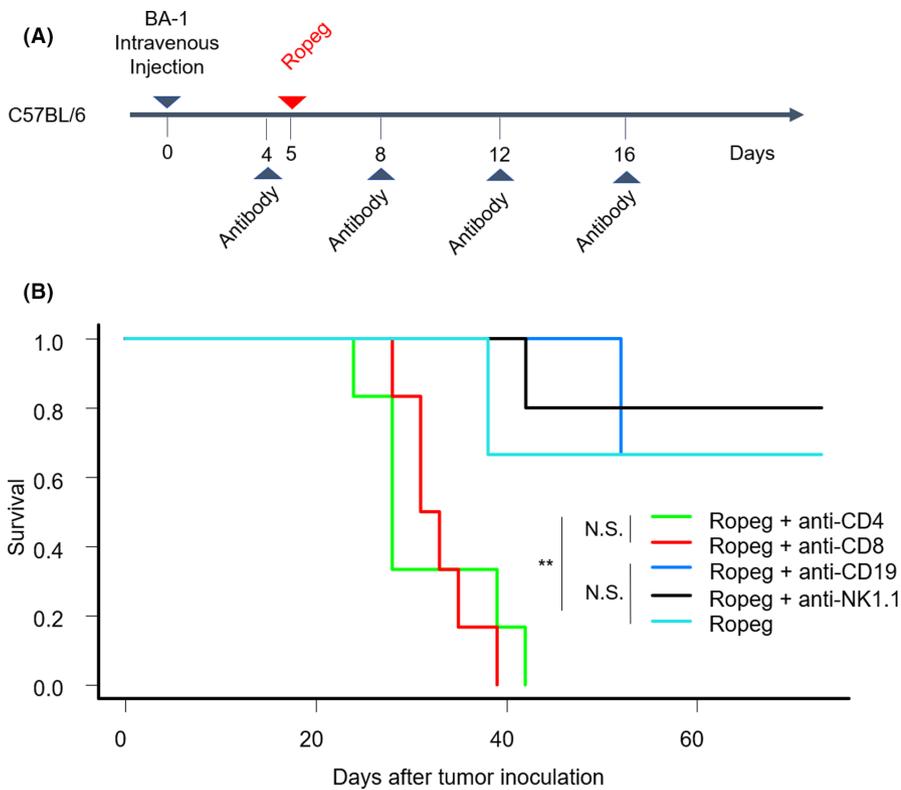
in treated wild-type mice. We therefore evaluated the efficacy of ropeg in C57BL/6 mice bearing BA-1 cells that were depleted of CD4<sup>+</sup>, CD8<sup>+</sup> T cells, NK1.1<sup>+</sup> cells, and CD19<sup>+</sup> B cells, respectively (Figure 6A). CD4<sup>+</sup> or CD8<sup>+</sup> T-cell depletion resulted in a significant loss of therapeutic efficacy (Figure 6B). Conversely,

B-cell and NK-cell depletion did not induce a loss of therapeutic efficacy (Figure 6B). These data show that the treatment efficacy of ropeg is dependent on adaptive immunity, with CD4<sup>+</sup> and CD8<sup>+</sup> T cells being essential mediators of the antitumoral immune response.

### 3.7 | Immunological memory is induced and T cells play a central role in mice that overcome leukemia after ropeg treatment

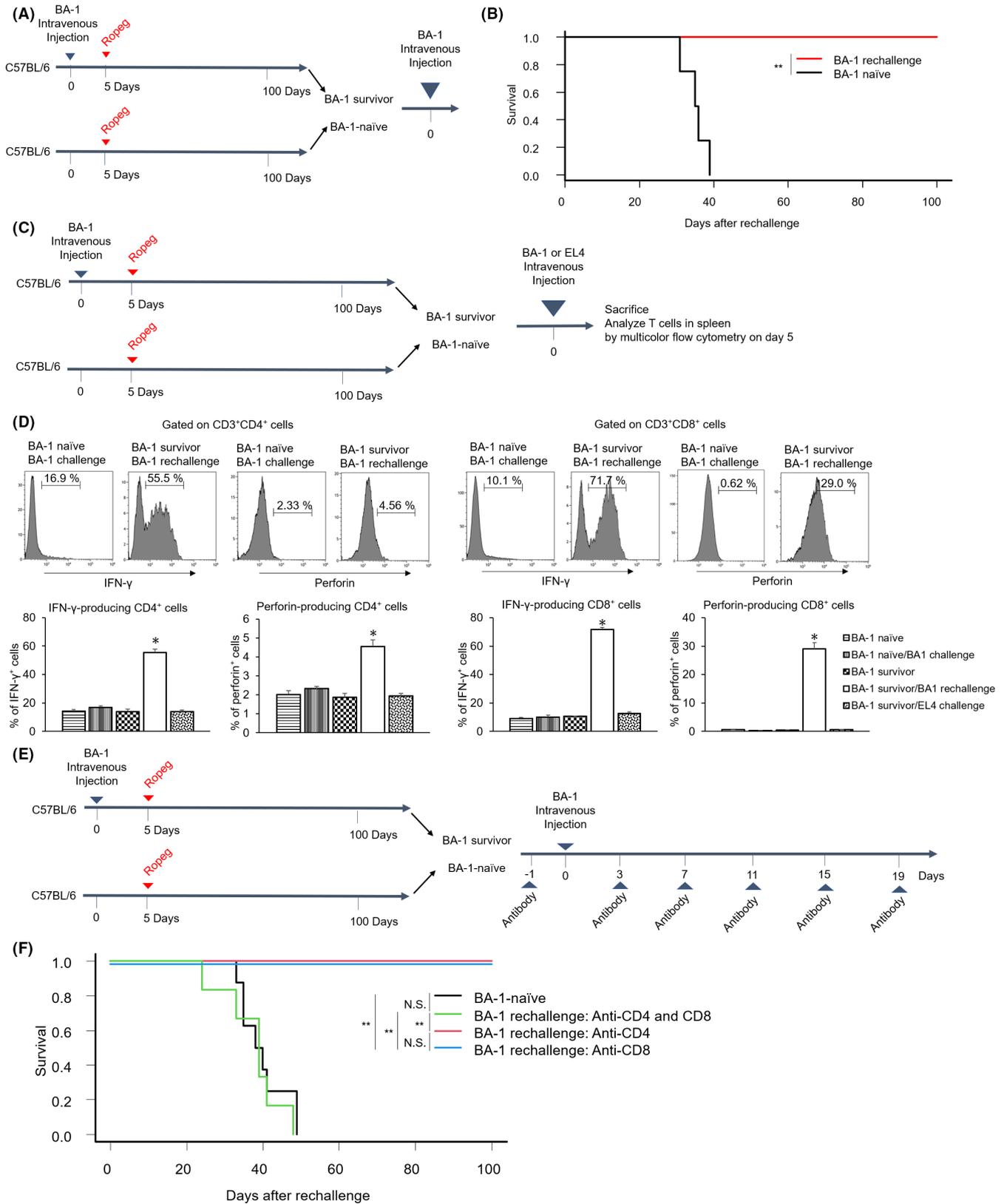
Next, we evaluated whether long-lasting immunological memory was established in ropeg-treated mice that had survived the BA-1

challenge. Surviving mice were rechallenged with BA-1 cells at least 100 days after the first BA-1 cell inoculation and compared with tumor-inoculated control mice (Figure 7A). Surviving mice withstood the BA-1 rechallenge in all cases (Figure 7B). This suggested that mice that overcame leukemia with ropeg treatment acquired an antitumor immune memory, and we further investigated whether this was an acquired immunity specific to BA-1 cells. BA-1-surviving mice with ropeg treatment produced a greater percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells producing cytotoxic cytokines compared with BA-1-naïve mice after the injection of BA-1 cells. Conversely, T cells from mice that had overcome BA-1 leukemia with ropeg treatment did not respond to intravenous infusion of EL4 cells (Figure 7C,D). These results suggest that ropeg treatment induces tumor-specific



**FIGURE 6** Ropeg-induced tumor rejection is mediated by cellular immunity. A, A schematic illustration of the treatment method in the depletion experiments. Depleting antibodies were administered as described in the “Materials and Methods” section. B, Survival data in C57BL/6 mice were plotted in a Kaplan-Meier survival curve, and statistical significance was calculated with the log-rank test. CD4<sup>-</sup> ( $n = 6$ ), CD8<sup>-</sup> ( $n = 6$ ), NK1.1<sup>-</sup> ( $n = 5$ ), and CD19-depleted ( $n = 5$ ) C57BL/6 mice were treated with ropeg on day 5 after BA-1 cell ( $1 \times 10^5$ ) inoculation. This experiment included control mice ( $n = 3$ ) that received ropeg without antibodies. Data were derived from three independent experiments. (\*\* $p < 0.01$ )

**FIGURE 7** Tumor rechallenge of surviving mice with BA-1 leukemia. A, C57BL/6 mice that achieved leukemia-free survival for more than 100 d ( $n = 4$ ; eight mice were injected intravenously with BA-1 cells, and four survived by ropeg treatment) after intravenous injection of BA-1 cells were rechallenged with BA-1 ( $1 \times 10^3$ ). No further treatment was applied. Age-matched mice that received no intravenous infusion of BA-1 cells and only ropeg administration ( $n = 4$ ) served as controls. B, Survival data derived from three independent experiments were plotted in a Kaplan-Meier survival curve. Statistical significance was calculated using a log-rank test (\*\* $p < 0.01$ ). C, To evaluate whether BA-1 leukemia-surviving mice have cytotoxic T cells specific for BA-1 cells, BA-1 cells ( $1 \times 10^3$ ) or another cell line, EL4 cells ( $1 \times 10^3$ ), were administered to BA-1 leukemia-surviving mice or age-matched BA-1-naïve mice. Five days after intravenous injection of the cell lines, spleens were collected and analyzed for cytotoxic cytokine secretion in T cells via flow cytometry. D, Representative histogram plots showing IFN- $\gamma$  or perforin expression gated on CD3<sup>+</sup>CD4<sup>+</sup> (left) and CD3<sup>+</sup>CD8<sup>+</sup> (right) populations. The histograms show the number of cells per channel (vertical axis) versus IFN- $\gamma$  or perforin (horizontal axis). Error bars show SE ( $n = 5-6$  each population). Data were derived from three independent experiments. Statistical analyses were performed using a Kruskal-Wallis test followed by Mann-Whitney  $U$  test with Bonferroni correction (\* indicates  $p < 0.05$  for a two-arm comparison between the BA-1-rechallenge group and each of the other groups). E, A schematic illustration to evaluate T-cell involvement in immunological memory for BA-1 cells. BA-1-surviving mice were treated with depleting antibodies according to the doses described in the “Materials and Methods” section twice a week for a total of 3 wk before the rechallenge of BA-1 cells ( $1 \times 10^3$ ). F, Survival data derived from three independent experiments were plotted in a Kaplan-Meier survival curve. Statistical significance was calculated using a log-rank test (\*\* $p < 0.01$ ). BA-1-naïve ( $n = 8$ ), CD4-depleted ( $n = 3$ ), CD8-depleted ( $n = 3$ ), and CD4<sup>-</sup> and CD8-depleted BA-1-surviving ( $n = 6$ ) mice. Data were derived from three independent experiments



immunological memory that is overcome after ropeg administration. Based on these results, we tested whether *in vivo* T-cell depletion abolishes antitumor immunological memory in BA-1-surviving mice (Figure 7E). BA-1-surviving mice which had only CD4<sup>+</sup> or CD8<sup>+</sup> T cells rejected tumor cells after BA-1 cell rechallenge. Depletion of

both CD4<sup>+</sup> and CD8<sup>+</sup> T cells was required for loss of immunological memory (Figure 7F). These data show that each of the CD4<sup>+</sup> or CD8<sup>+</sup> T cells has a crucial role in antitumor immunological memory and antitumor cytotoxicity in mice that have overcome tumors after ropeg treatment.

## 4 | DISCUSSION

Immunotherapy using IFN- $\alpha$  has been shown to rely on two mechanisms, namely a direct cytotoxic or cytostatic effect on the tumor cells via the induction of apoptosis and cell cycle arrest and the induction of a cellular antitumoral immune response.<sup>32,33</sup> Although ropeg, which is a novel long-acting pegylated IFN- $\alpha$ , has been confirmed to have superior results in myeloproliferative neoplasms, the mechanism remains unclear.<sup>16,18,34</sup> In this study, we showed that ropeg provided better survival benefit in comparison with conventional IFN- $\alpha$  in immunocompetent mice bearing multiple tumor cell lines such as BA-1 or EL4, which proliferate in mice without prior treatment. Considering the complicated effects of ropeg on tumor cells and immune cells, the mechanistic study of immunomodulation is limited by the availability of a suitable tumor model that can separate these two effects. To our knowledge, this is the first article to examine the mechanism by which ropeg exerts excellent antitumor effects in both direct and indirect manners.

Ropeg induced a time-dependent increase in 72 hours in caspase-3 and p21 expression in leukemia cell lines, which was followed by an increase in apoptosis and cell cycle arrest over time. By contrast, the effect of rIFN- $\alpha$  mostly reached a maximum after 24 hours. Additional stimulation with rIFN- $\alpha$  every 24 hours did not lead to an increase in annexin expression or G0/G1 phase of tumor cells over time. Several reports have shown that refractoriness to restimulation of rIFN- $\alpha$  in tumor cell lines occurs after 24 hours from the first injection of IFN- $\alpha$ .<sup>35,36</sup> By contrast, continuous stimulation can induce the positive feedback loop, leading to the priming effect.<sup>36</sup> Indeed, a previous study has shown that IFN- $\alpha$  preparation exerts direct tumoricidal effects due to the continuous delivery of IFN- $\alpha$  to tumor cells *in vivo*.<sup>10</sup> Each ropeg and rIFN- $\alpha$  treatment significantly prolonged survival in *lfnar1*<sup>-/-</sup> mice bearing BA-1 cells, in which the ropeg-treated group had a significantly longer survival than rIFN- $\alpha$  group. Pegylated interferon formulations, such as ropeg, are more suitable forms for acting directly on tumor cells.

Nevertheless, the absence of long-term remission of disseminated BA-1 leukemia and loss of efficacy to EL4 in *lfnar1*<sup>-/-</sup> mice indicate a direct cytotoxic effect of ropeg treatment on leukemia cells contributes to a small part of the survival-prolonging effect. Wild-type mice treated with ropeg rejected *lfnar1*<sup>-/-</sup> leukemic cells. These results indicate that long-term survival after the induction of remission requires an antitumor immune response after the administration of ropeg. As a factor explaining these phenomena, we confirmed a numerical increase in CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and B cells in ropeg-treated mice, which are key components of antitumor adaptive immunity.<sup>37</sup> Especially, cytotoxic CD8<sup>+</sup> T cells play a pivotal role in providing effective antigen-specific immunity against tumors,<sup>38,39</sup> and CD4<sup>+</sup> T cells primarily mediate antitumor immunity by providing help for CD8<sup>+</sup> CTL and antibody responses, as well as via the secretion of effector cytokines, such as IFN- $\gamma$  perforin, and under specific contexts, via direct cytotoxicity against tumor cells.<sup>40,41</sup> Ropeg caused a polyclonal increase in T cells that maintained their TCR $\beta$  repertoire. Increases in T<sub>SCM</sub> and T<sub>EM</sub> were particularly prominent

in both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets. Of note, CD4<sup>+</sup> T cells include subsets such as Treg cells and Th17 cells, and an increase in the number of CD4<sup>+</sup> T cells does not generally translate directly into increased antitumor efficacy. Nevertheless, we detected significantly increased production of IFN- $\gamma$  and perforin not only in CD8<sup>+</sup> T cells but also in CD4<sup>+</sup> T cells after ropeg treatment. Additionally, the participation of the cellular immune response for the observed antitumor response is supported by the fact that we identified CD4<sup>+</sup> and CD8<sup>+</sup> T cells to mediate the treatment response, whereas B and NK cells were dispensable. The mechanism by which ropeg causes such activation of the acquired immune system and an increase in immune cells is unclear. One likely possibility is the involvement of sustained delivery of IFN- $\alpha$  to immune cells as well as the direct delivery of IFN- $\alpha$  to tumor cells. First, in T cells, continuous IFN- $\alpha$  stimulation promotes T-cell proliferation and differentiation into CTL.<sup>42,43</sup> Second, the sustained delivery of IFN- $\alpha$  for macrophages stimulates the secretion of IL-12.<sup>44</sup> IL-12, a proinflammatory cytokine with potent tumor-suppressive activity, represents a promising candidate for combinatorial immunotherapy. IL-12 can directly support the persistent cytotoxic activity of T cells, as well as improve antigen presentation, mitigate against antigen-negative escape, and reshape endogenous immune inhibitory cells within the tumor microenvironment.<sup>45</sup> We also found that ropeg treatment induces an immunological memory. T cells also play a central role in the process of tumor rejection by immunological memory in tumor-surviving mice, and the presence of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the mice was sufficient for tumor rejection. Some studies demonstrated that CD4<sup>+</sup> T cells were capable of protecting the host against tumor challenge and that they could mediate complete tumor regressions independently of CD8<sup>+</sup> T cells.<sup>46-48</sup> T cells from BA-1-surviving mice responded excessively to BA-1 rechallenge and expressed cytotoxic cytokines, whereas they did not respond to EL4 inoculation. These results suggest that ropeg treatment induces both tumor-specific memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which independently have the potential to prevent the host from tumor relapse.

In summary, ropeg treatment induces an immune cell-mediated response in preclinical cancer models, leads to long-term survival by enhancing adoptive immunity in T cells, and finally establishes an immunological memory that is protective against tumor relapse. This research will help inform future clinical research that seeks to develop the most effective strategies to implement this highly effective therapeutic agent in the treatment of cancer patients.

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## DISCLOSURE

Hirohisa Nakamae was gifted ropeginterferon alfa-2b (mP1101) from Pharmaessentia. Masayuki Hino received scholarship from Chugai Pharmaceutical. Hideo Koh received research funding from Chugai Pharmaceutical. The remaining authors declare no competing financial interests.

## ETHICAL STATEMENT

Animal experiments were approved by the Osaka City University Animal Ethics Committee and performed according to the institutional guidelines of Osaka City University.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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