OPEN

Leukemia (2017) **31**, 1333–1339

www.nature.com/leu

ORIGINAL ARTICLE

Cirmtuzumab inhibits Wnt5a-induced Rac1 activation in chronic lymphocytic leukemia treated with ibrutinib

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Signaling via the B cell receptor (BCR) plays an important role in the pathogenesis and progression of chronic lymphocytic leukemia (CLL). This is underscored by the clinical effectiveness of ibrutinib, an inhibitor of Bruton's tyrosine kinase (BTK) that can block BCR-signaling. However, ibrutinib cannot induce complete responses (CR) or durable remissions without continued therapy, suggesting alternative pathways also contribute to CLL growth/survival that are independent of BCR-signaling. ROR1 is a receptor for Wnt5a, which can promote activation of Rac1 to enhance CLL-cell proliferation and survival. In this study, we found that CLL cells of patients treated with ibrutinib had activated Rac1. Moreover, Wnt5a could induce Rac1 activation and enhance proliferation of CLL cells treated with ibrutinib at concentrations that were effective in completely inhibiting BTK and BCR-signaling. Wnt5a-induced Rac1 activation could be blocked by cirmtuzumab (UC-961), an anti-ROR1 mAb. We found that treatment with cirmtuzumab and ibrutinib was significantly more effective than treatment with either agent alone in clearing leukemia cells *in vivo*. This study indicates that cirmtuzumab may enhance the activity of ibrutinib in the treatment of patients with CLL or other ROR1$^+$ B-cell malignancies.

Leukemia (2017) **31,** 1333–1339; doi:10.1038/leu.2016.368

INTRODUCTION

CLL cells depend on interactions with cells and soluble factors present in the tumor microenvironment for proliferation and survival.[1–3] Among the pathways that may support CLL proliferation and survival *in vivo*, BCR-signaling plays a prominent role.[4] Crosslinking of the BCR leads to phosphorylation of CD79α/β and Src family kinase LYN, resulting in the recruitment and activation of the tyrosine kinase Syk, which induces a cascade of downstream signaling events, leading to enhanced B-cell survival.[4] The importance of this cascade in CLL biology appears underscored by the clinical activity of small-molecule inhibitors of intracellular kinases, which play critical roles in BCR-signaling, such as SYK, phosphoinositide 3-kinase (PI3K) or Bruton's tyrosine kinase (BTK).[5–7] Ibrutinib is a small molecule inhibitor of BTK that has proven highly effective in the treatment of patients with CLL. However, despite having excellent clinical activity, ibrutinib generally cannot eradicate the disease or induce durable responses in the absence of continuous therapy.[7,8]

The failure of ibrutinib to induce complete responses could be because of alternative survival-signaling pathways, which are not blocked by inhibitors of BTK. One such pathway is that induced by signaling through ROR1, an oncoembryonic antigen expressed on CLL cells, but not on normal postpartum tissues.[9,10] We found that ROR1 could serve as a receptor for Wnt5a,[9] which could induce non-canonical Wnt-signaling that activates Rho GTPases, such as Rac1, and enhance leukemia-cell proliferation and survival.[11–13] Activation of Rac1 by Wnt5a could be inhibited by an anti-ROR1 mAb, cirmtuzumab, which is a first-in-class humanized monoclonal antibody currently undergoing evaluation in clinical trials for patients with CLL.[14]

We hypothesized that the effects of ibrutinib on blocking BCR-signaling might be offset by non-canonical Wnt-signaling via ROR1. If so, then inhibition of both ROR1- and BCR-signaling might have an enhanced anti-tumor effect. In this study, we investigated whether Wnt5a/ROR1 signaling was affected by treatment with ibrutinib and examined the activity of ibrutinib and cirmtuzumab on CLL cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Blood samples and animals

Blood samples were collected from CLL patients at the University of California San Diego Moores Cancer Center who satisfied diagnostic and immunophenotypic criteria for common B-cell CLL, and who provided written, informed consent, in compliance with the Declaration of Helsinki and the Institutional Review Board of the University of California San Diego (Institutional Review Board approval number 080918). PBMCs were isolated as described previously.[13] All experiments with all mice were carried out in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals, and University of California San Diego approved study protocol. All mice were age and sex matched. Details of assays for BTK-occupancy, calcium flux, Rac1 activation, cell proliferation, cell cycle and animal work are described in the Supplementary Materials and Methods Section.

RESULTS

Ibrutinib fails to inhibit Wnt5a-induced Rac1 activation in CLL

We examined the blood mononuclear cells of patients who were taking ibrutinib at the standard dose of 420 mg per day. Freshly isolated CLL cells had activated Rac1, which diminished over time in culture in serum-free media unless provided with exogenous

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Received 19 July 2016; revised 24 October 2016; accepted 4 November 2016; accepted article preview online 1 December 2016; advance online publication, 3 January 2017

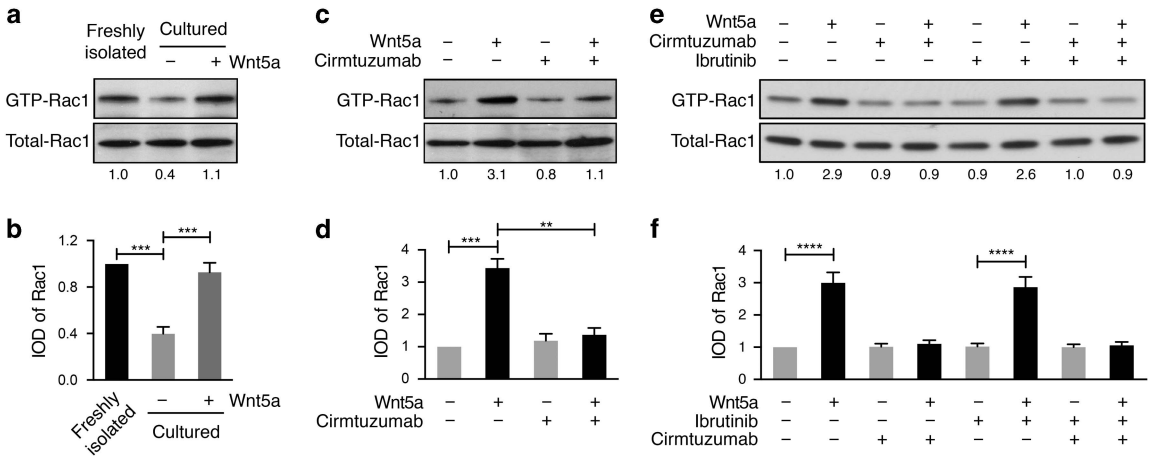

Figure 1. Cirmtuzumab inhibits Wnt5a-Induced Rac1 activation in ibrutinib-treated CLL cells. (**a**) Activated Rac1 was measured in the freshly isolated ibrutinib-treated CLL cells or isolated ibrutinib-treated CLL cells cultured in serum free media without or with exogenous Wnt5a (200 ng/ml), as indicated on the top of each lane. (**b**) Activated Rac1 was measured in the freshly isolated ibrutinib-treated CLL cells or isolated ibrutinib-treated CLL cells cultured in serum free media with or without Wnt5a (200 ng/ml). Mean Rac1 activation observed in four independent experiments is shown ($n = 4$). (**c**) CLL cells were collected from ibrutinib treated patients ($n = 4$). Activated Rac1 was measured in CLL cells treated with or without Wnt5a (200 ng/ml) or cirmtuzumab (10 μg/ml), as indicated above each lane of the immunoblot (**d**) Rac1 activation was measured in CLL cells collected from patients undergoing therapy with ibrutinib, which were treated with Wnt5a (200 ng/ml) and/or cirmtuzumab (10 μg/ml). The average Rac1 activation observed in five independent experiments is shown ($n = 5$). (**e**) Activated Rac1 was measured in CLL cells incubated with or without Wnt5a and treated with cirmtuzumab (10 μg/ml) or ibrutinib (0.5 μm), as indicated on the top of each lane. (**f**) Wnt5a-induced activation of Rac1 in CLL cells without treatment or treated with cirmtuzumab and/or ibrutinib. Mean Rac1 activation observed in five independent experiments is shown ($n = 5$). The numbers below each lane are ratios of band IOD of activated *versus* total GTPase normalized to untreated samples. Data are shown as mean ± s.e.m. for each group. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, as calculated using one-way ANOVA with Tukey's multiple comparisons test.

Wnt5a (Figures 1a and b), as noted for the CLL cells of patients not taking ibrutinib.[13] Moreover, the CLL cells from ibrutinib-treated patents were incubated with or without Wnt5a and/or cirmtuzumab. Immunoblot analysis showed that Wnt5a induced Rac1 activation in CLL cells from all patients examined, whereas treatment with cirmtuzumab inhibited Wnt5a-induced Rac1 activation (Figures 1c and d). These results indicate that therapy with ibrutinib does not inhibit ROR1-dependent, Wnt5a-induced Rac1 activation.

We examined whether treatment of CLL cells with ibrutinib *in vitro* could inhibit Wnt5a-induced Rac1 activation in CLL. For this, we incubated CLL cells collected from untreated patients with ibrutinib at concentrations of 0, 0.25, 0.5 or 1.0 μm for 2 h and then treated the cells with exogenous Wnt5a for 30 min. Immunoblot analysis demonstrated that ibrutinib could not block Wnt5a-induced Rac1 activation, even at ibrutinib concentrations of 1 μm (Supplementary Figure S1A), which is in large excess of what is required to achieve 100% occupancy of BTK and inhibition of BTK activity (Supplementary Figure S1B).[15–17] On the other hand, we noted that ibrutinib at concentrations as low as 0.25 μm inhibited the calcium flux induced by anti-IgM (Supplementary Figure S1C),[18] without acutely affecting CLL-cell viability (Supplementary Figure S1D).

The peak plasma concentration of ibrutinib in patients treated with this drug is ~0.5 μm, a concentration that can affect 100% occupancy and inhibition of BTK.[19,20] Therefore, ibrutinib was used at 0.5 μm for subsequent studies. We examined for Wnt5a-induced Rac1 activation with or without ibrutinib and/or cirmtuzumab. CLL cells were cultured with ibrutinib, cirmtuzumab or both ibrutinib and cirmtuzumab for 2 h, and then stimulated with exogenous Wnt5a for 30 min. For comparison, cells from the same CLL sample were cultured without Wnt5a in parallel. Treatment of CLL cells with Wnt5a induced activation of Rac1 to levels that were significantly higher than that of CLL cells that were not treated with Wnt5a (Figures 1e and f). Treatment with cirmtuzumab, but not ibrutinib, could inhibit Wnt5a-induced Rac1 activation in CLL cells (Figures 1e and f). As expected, ibrutinib did not block the capacity of cirmtuzumab to inhibit Wnt5a-induced Rac1 activation (Figures 1e and f).

Cirmtuzumab inhibits Wnt5a-enhanced proliferation of CLL cells treated with ibrutinib

Activation of Rac1-GTPase can enhance proliferation,[11,13,21] whereas loss of Rac1 results in impaired hematopoietic-cell growth.[22] We induced proliferation of CLL cells by co-culturing leukemia cells with HeLa cells expressing CD154 ($HeLa_{CD154}$) and recombinant interleukin (IL)-4 and IL-10.[13,23] Addition of exogenous Wnt5a to co-cultures of CLL cells with $HeLa_{CD154}$ cells and IL-4/10 significantly enhanced the proportion of dividing CLL cells. Treatment of the CLL cells with cirmtuzumab, but not ibrutinib, could block Wnt5a-enhanced proliferation in CLL cells (Figure 2a). The same effects were observed for CLL cells of different patients ($n = 6$) (Figure 2b). As expected, co-culturing with HeLa cells in the presence of IL4/10 and/or Wnt5a could not induce CLL-cell proliferation (Supplementary Figure S2).

We also performed cell-cycle analysis on permeabilized leukemia cells using propidium iodide and found that Wnt5a stimulation significantly enhanced the fraction of CD154-stimulated leukemia cells in S/G2/M (Figures 2c and d). The capacity of Wnt5a to enhance the proportion of cells in S/G2/M could be inhibited by treatment with cirmtuzumab, but not ibrutinib (Figures 2c and d). Collectively, these data demonstrate that cirmtuzumab could block Wnt5a-signaling leading to enhanced leukemia-cell proliferation, which was not affected by treatment with ibrutinib.

Activity of cirmtuzumab and/or ibrutinib in CLL patient-derived xenografts

We transferred CLL cells into the peritoneal cavity of immunodeficient $Rag2^{-/-}\gamma_c^{-/-}$ mice,[24] and examined whether treatment with ibrutinib and/or cirmtuzumab could deplete CLL cells *in vivo*. For this, we injected 1×10^7 viable primary CLL cells in AIM-V medium into the peritoneal cavity of each mouse. One day later, the mice were provided no treatment or daily doses of ibrutinib

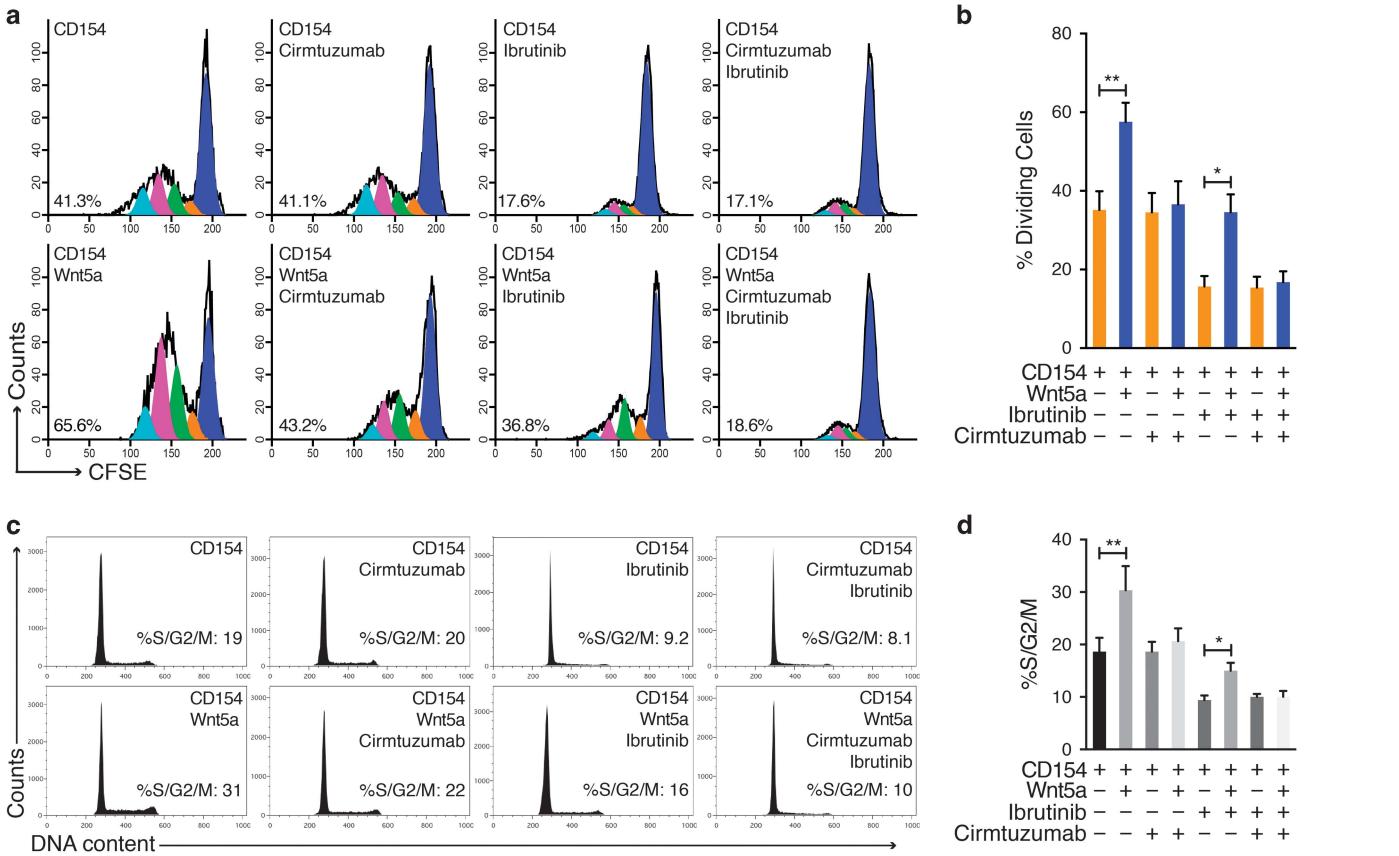

Figure 2. Cirmtuzumab inhibits Wnt5a-enhanced proliferation in ibrutinib-treated CLL Cells. (**a**) CD154-induced proliferation of CFSE-labeled CLL cells ($n = 6$) with or without Wnt5a and treated with cirmtuzumab (10 μg/ml) or ibrutinib (0.5 μm). One representative CLL sample is shown with the percent of dividing cells. (**b**) The bars indicate the mean proportions of CLL cells with diminished CFSE fluorescence from each of six different patients for each culture condition indicated at the bottom. (**c**) CLL cells were co-cultured on $HeLa_{CD154}$ in the presence of IL-4/10 or Wnt5a, and then treated with cirmtuzumab (10 μg/ml) or ibrutinib (0.5 μm) for 4 days, subjected to cell-cycle analysis following propidium iodide staining. One representative CLL sample is shown. (**d**) The mean fraction of cells in S/G2/M phase for all 4 patients tested is presented. Data are shown as mean ± s.e.m.; $*P < 0.05$; $**P < 0.01$, as determined by one-way ANOVA with Tukey's multiple comparisons test.

at 15 mg/kg via oral gavage, and/or a single dose of cirmtuzumab at 1 mg/kg via i.p. injection. After 7 days, the CLL cells were harvested via peritoneal lavage and the proportions of CLL cells in the harvested peritoneal cells were examined by flow cytometry (Figure 3a). The percentages and total numbers of CLL cells in peritoneal lavage were significantly lower in mice treated with cirmtuzumab or ibrutinib than in mice that did not receive any treatment. However, significantly fewer CLL cells were found in the peritoneal lavage of mice treated with cirmtuzumab and ibrutinib than in the peritoneal lavage of mice treated with either agent alone (Figure 3b).

Cirmtuzumab, but not ibrutinib, inhibits Wnt5a-enhanced Rac1 activation and proliferation of ROR1 × TCL1 leukemia cells

ROR1 × TCL1 leukemia cells were isolated from ROR1 × TCL1 double-transgenic mice that developed $ROR1^+$ leukemia.[10] We pretreated ROR1 × TCL1 leukemia cells with ibrutinib or cirmtuzumab for 2 h and then cultured the cells with or without Wnt5a for 30 min. Similar to our findings with human CLL cells, Wnt5a-induced Rac1 activation could be inhibited by cirmtuzumab, but not by ibrutinib (Figures 4a and b). The combination of cirmtuzumab with ibrutinib also inhibited Wnt5a-induced activation of Rac1 to levels observed in untreated cells (Figures 4a and b). However, Wnt5a treatment could not induce activation of Rac1 in the leukemia cells of single-transgenic TCL1 mice, which develop a leukemia that lacks expression of ROR1 (Supplementary Figure S3A).[10]

Again, we induced proliferation of ROR1 × TCL1 leukemia cells by co-culturing the cells with $HeLa_{CD154}$ in the presence of recombinant IL-4/10. Exogenous Wnt5a treatment significantly enhanced the percentage of numbers of cell divisions (Figure 4c). As with human CLL cells, Wnt5a and/or IL-4/10 alone could not induce proliferation of $ROR1^+$ leukemia cells of ROR1×TCL1 transgenic mice (Figure 4c), indicating a dependency on CD154 for this effect. In agreement with earlier studies,[13] Wnt5a did not enhance the proliferation of ROR1-negative TCL1-leukemia cells co-cultured with $HeLa_{CD154}$ cells and IL-4/10 (Supplementary Figure S3B), indicating a dependency on ROR1 for this effect. Treatment with ibrutinib could not inhibit the capacity of Wnt5a to enhance the proliferation of CD154-induced ROR1 × TCL1 leukemia-cell proliferation. On the other hand, cirmtuzumab blocked the capacity of Wnt5a to enhance ROR1 × TCL1 leukemia cells proliferation in response to CD154 and IL-4/10 (Figure 4c).

As noted for human CLL cells, cell-cycle analysis on permeabilized ROR1 × TCL1 leukemia cells using propidium iodide demonstrated that Wnt5a could increase the fraction of CD154-stimulated $ROR1^+$ leukemia cells in S/G2/M (Supplementary Figure S4). Moreover, the capacity of Wnt5a to enhance the fraction of $ROR1^+$ leukemia cells in S/G2/M could be inhibited by treatment with cirmtuzumab, but not ibrutinib (Supplementary Figure S4).

Treatment of immunodeficient mice engrafted with ROR1 × TCL1 leukemia with cirmtuzumab and/or ibrutinib

We examined the capacity of cirmtuzumab and/or ibrutinib to inhibit ROR1 × TCL1 leukemia cell engraftment in $Rag2^{-/-}\gamma_c^{-/-}$ mice, which were each engrafted with 2×10^4 ROR1 × TCL1 leukemia cells. These mice each received daily ibrutinib at 15, 5, 1.67 mg/kg via gavage, or a single dose of cirmtuzumab at 1, 3 or 10 mg/kg via intravenous injection. After 25 days, the animals were sacrificed and the spleen of each animal was examined. Ibrutinib (Supplementary Figure S5A) or cirmtuzumab (Supplementary Figure S5B) reduced the numbers of splenic leukemia cells in a dose-dependent manner. We selected the cirmtuzumab dose of 1 mg/kg and the daily dose of ibrutinib 5 mg/kg for combination studies. While the engrafted mice treated with cirmtuzumab or ibrutinib alone had significantly smaller spleens than the engrafted animals that did not receive any treatment, the mice treated with the combination of cirmtuzumab and ibrutinib had the greatest reductions in spleen size (Figure 5a). Furthermore, the mean proportion and number of leukemia cells in the spleen were significantly lower in mice treated with cirmtuzumab or ibrutinib compared with engrafted mice that did not receive treatment (Figures 5b and c). However, the engrafted animals that were treated with cirmtuzumab and ibrutinib had significantly lower proportions and numbers of leukemia cells per spleen than all other groups (Figures 5b and c).

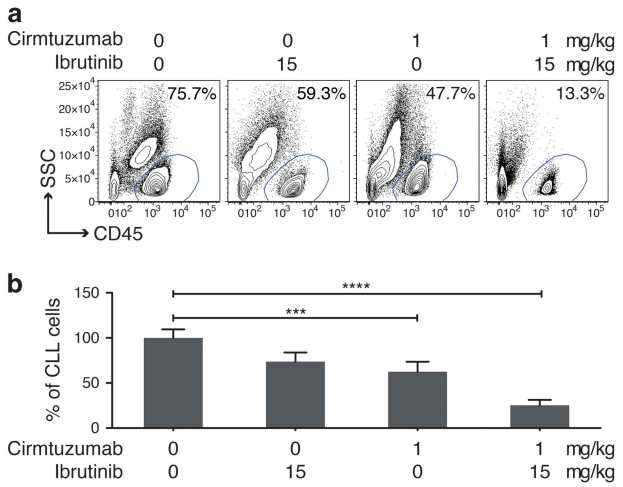

Figure 3. Effect of treatment with cirmtuzumab and/or ibrutinib on CLL patient derived xenografts. (**a**) CLL cells were injected to the peritoneal cavity of $Rag2^{-/-}\gamma_c^{-/-}$ mice 1 day before treatment. Peritoneal lavage was collected 7 days after cell injection and subjected to residual CLL determination by cell counting and flow cytometry analysis following staining with mAb specific for CD5, CD19 and CD45. The percentages shown in the top right of each contour plot indicates the proportion of CLL cells among the cells harvested from mice after treatment. (**b**) Each bar in the graph represents the percentage of CLL cells among harvested cells from mice after treatment, normalized with respect to the percentage of CLL cells among cells harvested from mice without treatment, which was to 100%. Data shown are mean ± s.e.m. from 3 different patients with 5 mice in each group; $^{***}P < 0.001$; $^{****}P < 0.0001$, as calculated using one-way ANOVA with Tukey's multiple comparisons test.

Treatment of immunocompetent mice engrafted with ROR1 × TCL1 leukemia with cirmtuzumab and/or ibrutinib

We examined the capacity of cirmtuzumab and/or ibrutinib to inhibit engraftment of ROR1 × TCL1 leukemia cells ($CD5^+$ $B220^{low}ROR1^+$) in immunocompetent human-ROR1 transgenic (ROR1-Tg) mice.[10] We injected 2×10^4 ROR1 × TCL1 leukemia cells to ROR1-Tg mice, and administered no treatment, daily doses of ibrutinib at 5 mg/kg via gavage, or weekly doses of cirmtuzumab at 10 mg/kg via intravenous injection. After 28 days, the animals were sacrificed and the spleen of each animal was examined. While the engrafted mice treated with cirmtuzumab or ibrutinib alone had significantly smaller spleens than the engrafted animals that did not receive any treatment, the mice treated with the combination of cirmtuzumab and ibrutinib had the greatest reductions in spleen size (Figure 6a). Furthermore, the mean proportion of splenocytes that were leukemia cells and the average absolute number of leukemia cells in the spleen were significantly lower in mice treated with either cirmtuzumab or ibrutinib than in mice that did not receive any treatment (Figures 6b and c). However, the engrafted animals that were treated with cirmtuzumab and ibrutinib had significantly lower proportions and numbers of leukemia cells per spleen than all other groups (Figures 6b and c).

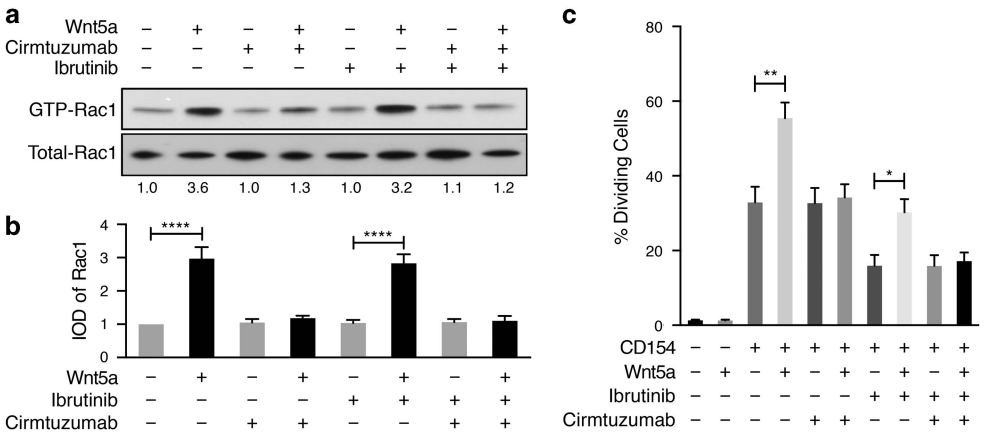

Figure 4. Cirmtuzumab inhibits Wnt5a-enhanced proliferation in ibrutinib-treated ROR1 × TCL1 leukemia cells. (**a**) Activated Rac1 was measured in ROR1 × TCL1 leukemia cells incubated with or without Wnt5a (200 ng/ml) and treated with cirmtuzumab (10 μg/ml) and/or ibrutinib (0.5 μm), as indicated on the top of each lane. The numbers below each lane are ratios of the band densities of activated *versus* total GTPase, normalized to untreated samples. (**b**) Activation of Rac1 in ROR1 × TCL1 leukemia cells treated with Wnt5a with or without cirmtuzumab (10 μg/ml) and/or ibrutinib (0.5 μm). The average Rac1 activation observed in five independent experiments is shown ($n = 5$). (**c**) CD154-induced proliferation of CFSE-labeled ROR1 × TCL1 leukemia cells ($n = 5$) with or without treatment with Wnt5a (200 ng/ml) and/or cirmtuzumab (10 μg/ml) or ibrutinib (0.5 μm). The bars indicate the mean proportions of ROR1 × TCL1 leukemia cells from each of five different mice that have diminished CFSE fluorescence for each culture condition, as indicated at the bottom. Data are shown as mean ± s.e.m.; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$, as calculated using one-way ANOVA with Tukey's multiple comparisons test.

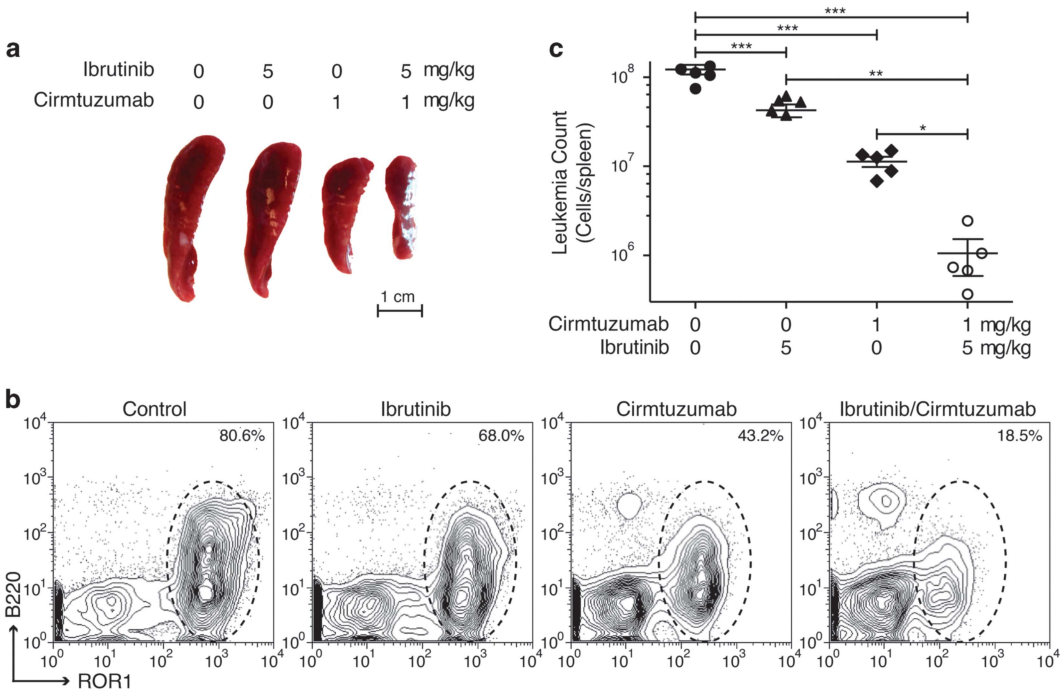

Figure 5. Additive inhibitory effect of treatment with cirmtuzumab and ibrutinib in immunodeficient mice engrafted histocompatible ROR1$^+$ leukemia. (**a**) Representative spleens of Rag2$^{-/-}\gamma_c^{-/-}$ mice were shown, which were collected 25 days after receiving an intravenous infusion of 2×10^4 ROR1×TCL1 leukemia cells. (**b**) Combination of cirmtuzumab and ibrutinib inhibits engraftment of ROR1×TCL1 leukemia cells in Rag2$^{-/-}\gamma_c^{-/-}$ mice. Rag2$^{-/-}\gamma_c^{-/-}$ mice were engrafted with 2×10^4 ROR1×TCL1 leukemia cells and then given single intravenous injection of 1 mg/kg cirmtuzumab on day 1, or daily does 5 mg/kg ibrutinib via oral gavage. Contour plots depicting the fluorescence of lymphocytes harvested on day 25 post adoptive transfer from representative mice ($n=5$) that received treatment, as indicated at the top, after staining the cells with fluorochrome-conjugated mAb specific for B220 (ordinate) and human ROR1 (abscissa). The percentages in the top right of each contour plot indicate the proportion of the blood mononuclear cells having CD5$^+$B220^{low}ROR1$^+$ phenotype of the leukemia cells. (**c**) Total number of ROR1×TCL1 leukemia cells in spleens of recipient Rag2$^{-/-}\gamma_c^{-/-}$ mice 25 days after adoptive transfer of 2×10^4 ROR1×TCL1 leukemia cells that received single injection of 1 mg/kg cirmtuzumab or daily injections of 5 mg/kg ibrutinib. Each symbol represents the number of leukemia cells found in individual mice. Data are shown as mean ± s.e.m. for each group of animals ($n=5$); *$P<0.05$, **$P<0.01$, ***$P<0.001$, as calculated using one-way ANOVA with Tukey's multiple comparisons test.

DISCUSSION

In this study, we examined the CLL cells of patients undergoing treatment with ibrutinib, which is highly effective at inhibiting BCR-signaling through its capacity to inhibit BTK.[25,26] First, we noted that the CLL cells of patients treated with ibrutinib had activated Rac1, which diminished over time in culture in serum-free media unless we supplemented the media with exogenous Wnt5a. Moreover, we found that Wnt5a could induce CLL to activate Rac1, as noted in a variety of cell types,[27–29] including CLL cells.[13] Subsequent studies showed that Wnt5a could induce Rac1 activation even in CLL cells that were treated with ibrutinib at supra-physiologic concentrations, which exceeded the levels required to achieve 100% occupancy and inhibition of BTK and BCR-signaling. In some respects this is similar to the findings of Ren and colleagues, who reported that ibrutinib could not inhibit FcγR-induced Rac1 activation, even though it could inhibit FcγR-induced calcium signaling and cytokine production.[17] However, the Wnt5a-signaling noted in our study was dependent upon ROR1, as indicated by the capacity of cirmtuzumab to inhibit Wnt5a-induced activation of Rac1. We conclude that ibrutinib cannot block ROR1-dependent, Wnt5a-induced activation of Rac1, which serves as an intracellular signal transducer that can influence multiple signaling pathways.

Activated Rac1 might mitigate the effectiveness of anti-cancer therapy. Prior studies found that activated Rac1 can enhance resistance of CLL cells to cytotoxic drugs.[11] One study found that activated T cells and fibroblasts could induce CLL cells to activate Rac1 and acquire resistance to the cytotoxic effects of fludarabine monophosphate; inhibition of activated Rac1 could restore the sensitivity of these CLL cells to this drug.[11] In another study, Rac1 was found to interact with and enhance the function of Bcl-2,[30] which is over-expressed in CLL.[31] Another study involving acute leukemia cells found that treatment with NSC-23766, an inhibitor of activated Rac1, could enhance the cytotoxicity of Bcl-2 antagonists for leukemia cells.[32] Finally loss of p53 in lymphoma cells has been associated with increased activation of Rac1, which could be inhibited by NSC-23766 or a dominant-negative form of Rac1, Rac1N17, leading to a dose-dependent increase in the rate of spontaneous or drug-induced apoptosis.[33] Conceivably the activated Rac1 observed in CLL cells of patients treated with ibrutinib provides an ancillary signal, which enhances the survival of leukemia cells of patients treated with ibrutinib.

Furthermore, Wnt5a-signaling also could promote leukemia-cell proliferation in patients treated with ibrutinib. The functional consequences of Wnt5-signaling in part are demonstrated by the ability of Wnt5a to enhance proliferation induced by CD154, which can induce CLL proliferation *in vitro* in the presence of exogenous IL4/10 or IL-21.[13,23,34] Although ibrutinib partially could inhibit CD154-induced CLL cell proliferation, possibly because of its capacity to inhibit BCR and BCR-independent pathways,[25,26,35] we found that ibrutinib could not inhibit the capacity of Wnt5a to enhance CD154-induced CLL proliferation via ROR1-dependent signaling, which could, however, be blocked by treatment with cirmtuzumab.

Wnt5a most likely is produced by cells in the CLL microenvironment. However, prior studies found that plasma samples of patients with CLL also have high levels of Wnt5a relative to those of healthy adults.[13] Wnt5a also might be produced by the CLL cells

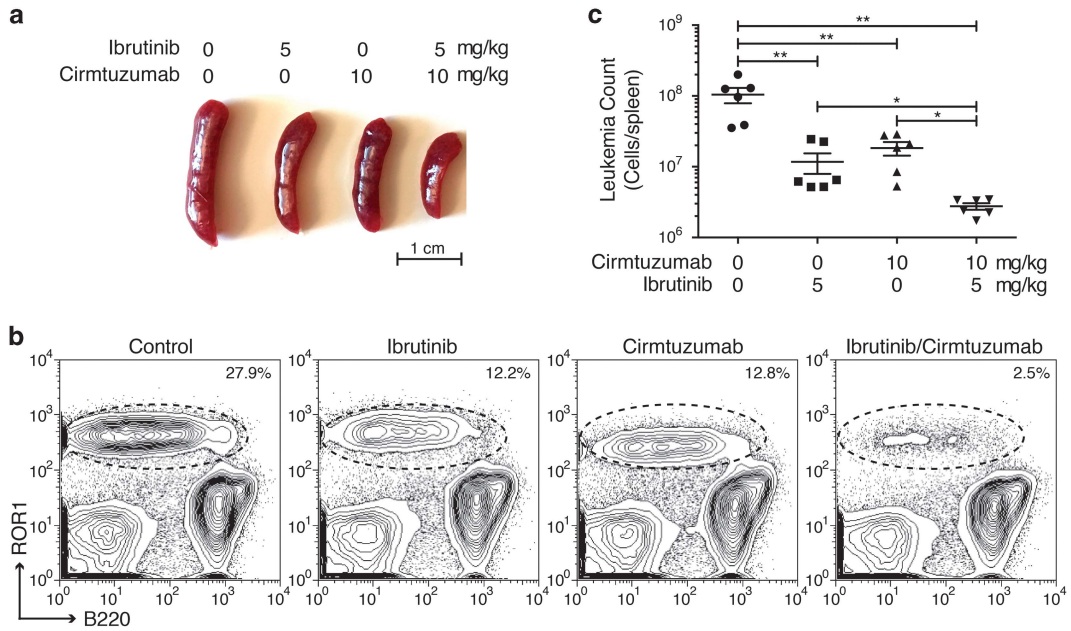

Figure 6. Additive inhibitory effect of treatment with cirmtuzumab and ibrutinib in immunocompetent mice engrafted histocompatible ROR1$^+$ leukemia. (**a**) Representative spleens of ROR1-Tg mice were shown, which were collected 25 days after receiving an intravenous infusion of 2×10^4 ROR1×TCL1 leukemia cells. (**b**) Combination of cirmtuzumab and ibrutinib inhibits engraftment of ROR1×TCL1 leukemia cells in ROR1-Tg mice. ROR1-Tg mice were engrafted with 2×10^4 ROR1×TCL1 leukemia cells and then given weekly intravenous injection of 10 mg/kg cirmtuzumab or daily does 5 mg/kg ibrutinib via oral gavage. Contour plots depicting the fluorescence of lymphocytes harvested 25 days after adoptive transfer of representative mice ($n=6$) that received treatment, as indicated at the top, after staining the cells with fluorochrome-conjugated mAb specific for B220 (abscissa) and human ROR1 (ordinate). The percentages in the top right of each contour plot indicate the proportion of the blood mononuclear cells having CD5$^+$B220^{low}ROR1$^+$ phenotype of the leukemia cells. (**c**) Total number of ROR1×TCL1 leukemia cells in spleens of recipient ROR1-Tg mice 28 days after adoptive transfer of 2×10^4 ROR1×TCL1 leukemia cells that received weekly injection of 10 mg/kg cirmtuzumab and/or daily doses of ibrutinib (at 5 mg/kg). Each symbol represents the number of leukemia cells found in individual mice. Data are shown as mean ± s.e.m. for each group of animals ($n=6$); *$P<0.05$, **$P<0.01$, as calculated using one-way ANOVA with Tukey's multiple comparisons test.

themselves, allowing for autocrine activation.[36] Indeed, one study found that CLL cells that express high levels of Wnt5a apparently have increased motility and chemotactic responses than CLL cells that express little or no Wnt5a, presumably due to the effects of Wnt5a-autocrine signaling.[36] We also noted in an earlier study that Wnt5a could enhance the migration of CLL cells toward chemokine via activation of RhoA.[13] However, because BTK plays a prominent role in CLL signaling via chemokine receptors such as CXCR4,[37] we focused our attention on the capacity of Wnt5a to activate Rac1, which could enhance proliferation induced by CD154 via signaling pathways that are relatively independent of BTK.

Because the Wnt5a-ROR1 signaling pathway appears intact in CLL cells treated with ibrutinib, we examined for additive, if not synergistic, effects of treatment with ibrutinib and cirmtuzumab. For mice engrafted with histocompatible ROR1$^+$ leukemia, or human CLL xenografts, we found that treatment with both cirmtuzumab and ibrutinib was significantly more effective than treatment with either agent alone in clearing leukemia cells *in vivo*. This study indicates that cirmtuzumab may enhance the activity of ibrutinib in the treatment of patients with CLL or other ROR1$^+$ B-cell malignancies.

Combination therapies are often more effective in treating patients with cancer.[38] Investigations are ongoing to evaluate the activity of ibrutinib in combination with other drugs, such as venetoclax or anti-CD20 mAbs.[39,40] However, other agents that target signaling pathways that are relatively independent of those influenced by BCR-signaling also could make excellent candidates for use in combination with ibrutinib. Because cirmtuzumab and ibrutinib target independent signaling pathways, they have apparent synergistic effects in clearing leukemia cells from our mouse models. By targeting more than one signaling pathway leading to leukemia-cell growth/survival, combined therapy with cirmtuzumab and ibrutinib also could potentially mitigate the risk of acquiring resistance to inhibitors of BTK, as sometimes occurs in patients who receive ibrutinib monotherapy.[41]

Taken together, from the perspective of therapeutic efficacy and drug resistance, our preclinical observations provide a rationale for the combination therapy with cirmtuzumab with ibrutinib, or other inhibitors of BTK such as acalabrutinib,[42,43] for patients with CLL or other B-cell malignancies that express ROR1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Brian Lannutti for technical support. We thank California Institute for Regenerative Medicine (CIRM) grant (DR3-06924) for supporting us in the generation of the anti-ROR1 mAb, cirmtuzumab. This work was supported by the UC San Diego Foundation Blood Cancer Research Fund (BCRF), a SPORE grant (7005-14) from the Leukemia and Lymphoma Society, and a PO1 grant (5P01CA081534-14) from the NIH for the CLL Research Consortium.

AUTHOR CONTRIBUTIONS

JY, LC and TJK conceived the project. JY, LC and TJK wrote the manuscript. JY, LC, BC, CW, MYC, YC, LZ, LZR and GFW performed the experiments. JY, LC and TJK analyzed the data.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)