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Localized Store-Operated Calcium Influx Represses CD95-Dependent Apoptotic Effects of Rituximab in Non-Hodgkin B Lymphomas

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The anti-CD20 mAb, rituximab, is routinely used to treat B cell malignancies. However, a majority of patients relapse. An improvement in the complete response was obtained by combining rituximab with chemotherapy, at the cost of increased toxicity. We reported that rituximab induced the colocalization of both the Orai1 Ca2+ release-activated Ca2+ channel (CRAC) and the endoplasmic reticulum Ca2+ sensor stromal interaction molecule 1 with CD20 and CD95 into a cluster, eliciting a polarized store-operated Ca2+ entry (SOCE). We observed that blocking this Ca2+ entry with downregulation of Orai1, pharmacological inhibitors, or reducing calcemia with hypocalcemic drugs sensitized human B lymphoma cell lines and primary human lymphoma cells to rituximab-induced apoptosis in vitro, and improved the antitumoral effect of rituximab in xenografted mice. This revealed that Ca2+ entry exerted a negative feedback loop on rituximab-induced apoptosis, suggesting that associating CRAC channel inhibitors or hypocalcemic agents with rituximab may improve the treatment of patients with B cell malignancies. The calcium-dependent proteins involved in this process appear to vary according to the B lymphoma cell type, suggesting that CRAC-channel targeting is likely to be more efficient than calcium-dependent protein targeting. 

The action mechanism of RTX mainly involves Ab-dependent cellular toxicity and complement activation, as well as direct induction of apoptosis (4). RTX-induced apoptosis is mediated by a complex signaling pathway, including activation or downregulation of protein kinases, phosphatases, and Bcl-2 family members, as well as changes in lipid distribution in the cell membrane, triggering mitochondrial- and CD95-dependent cell death (5). However, it has been established that RTX induces only modest levels of apoptosis in NHL B. Previous works have demonstrated that anti-CD20 mAb ligation to CD20 drives a rapid, sustained increase in the intracellular Ca2+ concentration ([Ca2+]i), relying on both intracellular and extracellular calcium pools (6–8). However, the molecular components responsible for this Ca2+ response, as well as its role in RTX activity, still remained elusive (8–10).

Calcium ions (Ca2+) act as second messengers in cell signaling, triggering various cellular processes, including gene transcription, secretion, cell proliferation, migration, and apoptosis (11). Intensity and spatially-temporally confined Ca2+ signaling constitutes a calcium signature, which may, potentially, trigger a specific, singular, cellular process (12–14). In nonexcitable cells, Ca2+ responses occur mainly through a biphasic signal, involving intracellular Ca2+ mobilization from the endoplasmic reticulum (ER), followed by a sustained Ca2+ entry across the plasma membrane (15). Store-operated Ca2+ entry (SOCE) is a major Ca2+ influx pathway in nonexcitable cells (16). By definition, SOCE is activated by Ca2+ release from the ER. Two genes are responsible for SOCE activity: stromal interaction molecule 1 (STIM1) functions as an ER Ca2+ sensor that detects store depletion (17), and Orai1 constitutes the pore-forming subunit of Ca2+ release-activated Ca2+ (CRAC) channels (18). Once ER-Ca2+ is depleted, STIM1 proteins aggregate and translocate to the close vicinity of the plasma membrane, activating CRAC channels and Ca2+ entry (17).

Abbreviations used in this article: [Ca2+]i, intracellular Ca2+ concentration; CRAC, Ca2+ release-activated Ca2+ channel; DLBCL, diffuse large B cell lymphoma; ER, endoplasmic reticulum; FL, follicular lymphoma; IP3, inositol 1,4,5-trisphosphate; NHL, non-Hodgkin lymphoma; PKC, protein kinase C; RTX, rituximab; shNT, non-targeting shRNA; shOrail, Orai1 shRNA; shRNA, short hairpin RNA; SOCE, store-operated Ca2+ entry; STIM1, stromal interaction molecule 1.
The present work revealed that the rituximab-induced Ca\textsuperscript{2+} response involved intracellular Ca\textsuperscript{2+} mobilization and subsequent Ca\textsuperscript{2+} entry due to CRAC channel activation. We demonstrated that this Ca\textsuperscript{2+} entry exerted a negative feedback loop on CD95-dependent RTX-induced apoptosis, and that Orai1 inhibition greatly reduced tumor growth in xenografted mice. These data suggest that associating CRAC inhibitors with RTX is likely to improve the treatment of patients with B cell malignancies.

Materials and Methods

Abs and reagents

Rituximab and trastuzumab were provided by Institut Bergonié Pharmacy Department. ML9, Hoechst 33258, and Xestospongion C were purchased from Sigma-Aldrich (L’Isle d’Abeau, France). BTP2 was from Interchim (Montluçon, France). Gö6976 was purchased from Calbiochem (Merck Millipore, Fontenay sous bois, France). FuraPE3-AM and fluo-8-AM were from Euromedex (Mundolsheim, France). FAM-Flica in vitro caspase 3, 8, and 9, and detection kits were purchased from AbD Serotec (Kidlington, U.K.). Anti-CD95 mAb (clone APO1-3) was purchased from Enzo Life Sciences (Villeurbanne, France). The anti-protein kinase C (PKC) \(\alpha\) clone (clone C-20), anti-PKC\(\beta\) clone (clone C-16), and anti-PKC\(\beta\) clone (clone C-18) rabbit polyclonal Abs were supplied by Santa Cruz Biotechnology (Heidelberg, Germany). Anti-rituximab FITC mAb (clone MB2 A4) was supplied by AbD Serotec (Bio-Rad, Colmar, France). The anti-CD19 PE (clone HIB 19) and anti-CD20 FITC (clone 2H7) mAbs were from eBiosciences (San Diego, CA). The anti-human Orai1 and STIM1 rabbit polyclonal Abs were from Alomone Laboratories (Jerusalem, Israel). Alexa 488– or 594–conjugated donkey anti-mouse and Alexa 488– or 594–conjugated donkey anti-rabbit polyclonal Abs came from Life Technologies (Saint Aubin, France).

Short hairpin RNA lentiviral transduction

The short hairpin RNA (shRNA) lentivirus transduction approach was used to knock down \(orai1\) gene expression. Two pLKO1 lentiviral vectors expressing DNA sequences encoding for Orai1 \(96\) shRNA (TRCN0000439906) (shOrai1 \(96\)) and Orai1 \(44\) shRNA (TRCN0000165044) (shOrai1 \(44\)) were purchased from Sigma-Aldrich. The pX508 nontargeting shRNA (shNT) was used as lentivirus cell transduction control. Lentiviruses were produced by Santa Cruz Biotechnology (Heidelberg, Germany).

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Patient samples and cell line cultures

Subjects were recruited under the Rennes University and Institut Bergonié Institutional Review Board approval and informed consent process, in accordance with the Declaration of Helsinki. Lymph nodes were collected from follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) patients. Clinical characteristics of patients are described in Table I. Cell suspensions were obtained, as previously described (19). Before experiments, dead cells were discarded using the dead-cell removal kit from Miltenyi Biotec (Paris, France), whereas live cells were cultured in RPMI 1640 (Invitrogen), supplemented with 10% FCS and penicillin/streptomycin (Invitrogen). The Raji Burkitt lymphoma and SUDHL4 FL-transformed cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH cell collection (Braunschweig, Germany).

Calcium imaging

Single-cell [Ca\textsuperscript{2+}]i imaging was performed ratiometrically, using FuraPE3-AM calcium as described previously (20). Cells were loaded with 5 \(\mu\)M FuraPE3-AM at room temperature in HBSS for 30 min. FuraPE3-AM exhibits limited compartmentalization in intracellular stores and is leakage resistant (21). The cells were rinsed with HBSS and incubated in the absence of the Ca\textsuperscript{2+} probe for 15 min to complete de-esterification of the dye. Fluorescence micrograph images were captured at 510 nm, using an inverted epifluorescence microscope (Olympus IX70) equipped with a ×40 L’Aapo341-1.5W objective. FuraPE3-AM was excited at 340 and 380 nm alternately, and the ratios of the resulting images were produced at constant 10 s intervals. For experiments on anti-RTX FITC– or CD20 FITC–treated cells, Fluo8-AM was used instead of FuraPE3-AM, as FITC fluorescence disturbs Ca\textsuperscript{2+} measurements with FuraPE3. Anti-CD20 FITC and RTX FITC localizations were determined by their fluorescence emission at 530 nm for a light excitation at 485 nm. Ca\textsuperscript{2+} changes were evaluated by exciting Fluor at 535 nm and measuring the emitted fluorescence at 605 nm. As in the case of FuraPE3-AM, cells were loaded with Fluor8-AM (2 \(\mu\)M) in HBSS for 30 min and then incubated in Ca\textsuperscript{2+} probe-free HBSS for 15 min to complete de-esterification of the dye. Regions of interest were drawn on certain recorded cells to restrict data collection to specific regions. Imaging was controlled by Universal Imaging software, including Metafluor and Metamorph. Data were processed using OriginPro 7.5 software (Origin Lab).

Confocal microscopy

Cells were untreated or treated with RTX (10 \(\mu\)g/ml) for 15 min and fixed in PBS containing 4% w/v paraformaldehyde at 4°C for 10 min. Cells were then permeabilized in PBS supplemented with 5% BSA/0.1% saponin for 5 min. Cells were incubated with anti-CD95 mAb (APO1-3) or anti-RTX FITC and anti-ORAI1 or anti-STIM1 Abs in PBS/1% w/v BSA at room temperature for 60 min. CD95 was revealed using secondary Alexa 594–conjugated donkey anti-mouse Ab and ORAI1 or STIM1 using Alexa 488– or Alexa 594–conjugated donkey anti-rabbit Ab. Nuclei were stained using Hoechst 33258. Images were acquired using a Zeiss LSM 510 meta confocal microscope (Zeiss, Göttingen, Germany) with an ApoPLAN ×63 objective.

Apoptosis assay

B cell lines or primary lymphoma B cells were untreated or pretreated with ML9 or BTP2 for 1 h. Cells were then incubated with or without rituximab for 24 h. B cell apoptosis was evaluated as the percentage of cells with active caspase 8, 9, or 3, detected by the FAM-Flica in vitro caspase detection kit, used according to the manufacturer’s instructions. Apoptotic cells were analyzed by flow cytometry using a FACSCalibur cytometer and Cell Quest software (BD Biosciences). For primary FL B cells, apoptotic cells were analyzed on selectively gated CD19-positive/active caspase-3–positive cells.

In vivo experiments

In vivo experiments were performed according to ethical criteria approved by the Ministère de l’Enseignement Supérieur et de la Recherche (agreement n° B33-522-2, authorization n° 00892-01).

Five million SUDHL4 cells or shORAI1- or shNT-expressing SUDHL4 cells were harvested, suspended in Matrigel (BD Biosciences), and injected s.c. into the dorsal flank of 8-wk-old female Ragy2C2–/– mice (22), shORAI1- or shNT-derived tumors were treated with vehicle or RTX (0.01 mg/kg) by i.p. injection three times per week. SUDHL4-derived tumors were treated i.p. either with vehicle or RTX (0.01 mg/kg) and/or a SOCE inhibitor, BTP2 (12 \(\mu\)g/kg) or ML9 (50 \(\mu\)g/kg). The treatment schedule was as follows: injection three times per week with vehicle or SOCE inhibitors, followed by injection with RTX throughout the experiment until the mice were sacrificed. For zoledronate experiments, female Ragy2C2–/– mice (n = 8/group) were xenografted s.c. into the dorsal flank with one million Raji cells. Mice were injected i.p. three times per week with vehicle, zoledronate (1 mg/kg), RTX, or RTX plus zoleodronate throughout the experiment until the mice were sacrificed. In combination, zoledronate was systematically injected 2 h before RTX. Tumor volumes were determined by measuring the length (l) and width (w) of the tumors with a caliper and calculating the volume using the following formula: \(V = lw^2/2\).

Statistical analysis

The significance of differences was calculated using the \(t\) test or one-way ANOVA test, as appropriate (\(*p < 0.05\).

Results

Calcium responses induced by RTX

The addition of RTX provoked a cytosolic Ca\textsuperscript{2+} increase, consisting of a peak followed by a sustained plateau phase in SUDHL4 and Raji cells (Fig. 1Aa, Supplemental Fig. 1Aa), whereas no calcium increase was observed with the irrelevant IgG1 anti-HER2 mAb trastuzumab (data not shown). Cells recorded in Ca\textsuperscript{2+}–free medium exhibited only the Ca\textsuperscript{2+} peak, whereas the plateau phase was abrogated (Fig. 1Aa, Supplemental Fig. 1Aa). The biphasic response was, therefore, due to the mobilization of intracellular Ca\textsuperscript{2+} stores.
FIGURE 1. Rituximab induced colocalization of Orai1, STIM1, and CD20 in a cluster, eliciting a localized Ca\(^{2+}\) influx in the SUDHL4 cell line. (A) RTX provokes intracellular Ca\(^{2+}\) responses. Ca\(^{2+}\) responses to RTX (10 \(\mu\)g/ml) were measured ratiometrically in Fura-PE3–loaded cells. Black arrows indicate RTX addition. Recordings were performed at 37°C with a conventional videomicroscopy setup (Olympus IX-70 microscope, objective \(\times 40\)). Data was processed using OriginPro 7.5 software (Origin Lab). The data represent mean ± SE of three independent experiments. (Aa) Cells were recorded in extracellular medium containing 2 mM Ca\(^{2+}\) (control, in black, \(n = 50\)) or Ca\(^{2+}\)-free medium (0 mM Ca\(^{2+}\), in red, \(n = 98\)). (Ab) Cells were preincubated or not (control, in black, \(n = 98\)) with Xestospongin C (360 nM, in blue, \(n = 74\)) or 2-aminoethoxydiphenyl borate (44 \(\mu\)M, in red, \(n = 40\)) for 15 min and recorded in Ca\(^{2+}\)-free medium. (Ac) Cells were preincubated or not (control, in black, \(n = 45\)) with ML9 (50 \(\mu\)M, in blue, \(n = 59\)) or BTP2 (10 \(\mu\)M, in green, \(n = 18\)) for 15 min and recorded in extracellular medium containing 2 mM Ca\(^{2+}\). (Ad) Calcium responses to RTX recorded in cells expressing shNT (in black, \(n = 51\)), shOrai1 096 (in red, \(n = 69\)), or shOrai1 044 (in green, \(n = 59\)). Recordings were performed in extracellular medium containing 2 mM Ca\(^{2+}\). Inset, SUDHL4 cells infected with nontargeting or two different Orai1-targeting shRNA lentiviruses were lysed, and the expression level of Orai1 was evaluated by Western blot. Beta-actin was used as a loading control. (B) Ca\(^{2+}\) influx colocalized with CD20 capping. (Ba) SUDHL4 cells, treated with RTX (10 \(\mu\)g/ml) at 37°C for 15 min, were stained with anti-RTX FITC, and then Ca\(^{2+}\) recordings were performed. (Bb) Cells, treated with thapsigargin (100 nM) at 37°C for 15 min, were stained with anti-CD20 FITC. CD20 and RTX labeling was analyzed using the same conventional videomicroscopy setup. Fluo8 fluorescence images were obtained every 10 s and translated into false colors, according to the color scale shown on the right of the recorded cells. Cells were bathed in a Ca\(^{2+}\)-free extracellular medium (white bar) containing thapsigargin (100 nM) (control) or not (RTX), and 2 mM Ca\(^{2+}\)-containing extracellular medium was perfused in the bath (black bar) to visualize SOCE. Intracellular Ca\(^{2+}\) concentrations were recorded at several time points for each condition. Black arrowheads and numbers on the diagram correspond to the annotated images above. White arrow indicates CD20 capping. On the diagrams, colored curves show the Fluo8 fluorescence recorded in corresponding colored region of interest drawn in the cells. (C) Representative images of immunostaining showing colocalization of Orai1 and STIM1 with CD20 capping. SUDHL4 cells were incubated in the absence or presence of RTX (10 \(\mu\)g/ml) at 37°C for 15 min. Cells were fixed and stained with anti-CD20 FITC or anti-RTX FITC and anti-Orai1 or anti-STIM1 revealed by donkey anti-rabbit Ab coupled to Alexa 594. Nuclei are depicted in blue. Images were acquired with a Zeiss LSM510 confocal microscope using an Apoplan \(\times 63\) objective.
and extracellular Ca\textsuperscript{2+} influx. To determine the origin of the intracellular Ca\textsuperscript{2+} store mobilization, cells were pretreated with Xestospongin C or 2-aminoethoxydiphenyl borate, two inositol 1,4,5-trisphosphate (IP3) receptor inhibitors, and recorded in Ca\textsuperscript{2+}-free medium. Under these conditions, the RTX-induced intracellular Ca\textsuperscript{2+} store mobilization was dramatically reduced, suggesting the involvement of an IP3-dependent Ca\textsuperscript{2+} release from ER (Fig. 1Ab, Supplemental Fig. 1Ab). We next investigated whether CRAC channels participated in the Ca\textsuperscript{2+} influx triggered by RTX. Treating SUDHL4 and Raji cell lines with BTP2 or ML9, two CRAC channel blockers, abolished the sustained Ca\textsuperscript{2+} response, but did not affect the initial intracellular Ca\textsuperscript{2+} mobilization (Fig. 1Ac, Supplemental Fig. 1Ac). SUDHL4 cells stably expressing a shRNA Orai1 (shOrai1 044 and shOrai1 096) were generated to test whether Orai1, the pore-channel subunit of CRAC channels in lymphocytes, participated in the RTX-induced Ca\textsuperscript{2+} entry. In Orai1 knockdown cells, the RTX-induced plateau phase Ca\textsuperscript{2+} response was inhibited compared with cells expressing shNT, whereas the first phase of intracellular Ca\textsuperscript{2+} mobilization was unchanged (Fig. 1Ad). Moreover, single-cell Ca\textsuperscript{2+} imaging experiments, adding back Ca\textsuperscript{2+} to cells bathed in a Ca\textsuperscript{2+}-free medium and prestimulated with 10 \mu g/ml RTX, revealed that the resulting Ca\textsuperscript{2+} influx was initially localized in a cell region exhibiting an accumulation of CD20–RTX complex, a phenomenon we called CD20 capping (15.2 \pm 1.2\% of cells for STIM1) (Supplemental Fig. 1B).

Inhibitors or decreasing extracellular Ca\textsuperscript{2+} concentrations may enhance the antitumoral effect of RTX. To verify this hypothesis, Rag2\textsuperscript{−/−}mice were xenografted with SUDHL4 cells down-expressing Orai1 and treated three times per week with RTX or vehicle. The growth of shOrai1-expressing cells under control conditions was initially observed to be dramatically delayed (shOrai1 096) or even inhibited (shOrai1 044) compared with that of shNT-expressing cells. Interestingly, these experiments revealed that the antitumoral effect of RTX increased significantly in mice bearing shOrai1 096–expressing cells compared with shNT-expressing cells (Fig. 4A). This result was confirmed by pharmacological treatment of mice xenografted with SUDHL4 cells and treated three times per week with RTX, alone or in combination with ML9 or BTP2. Mice treated with RTX and ML9 or BTP2 exhibited a significant reduction in tumor volume, compared with drug treatment alone (Fig. 4B). Hypocalcemic agents, such as zoledronate, are currently used in cancer treatment. Although zoledronate has been reported to directly induce apoptosis in various neoplastic cells (27), it was unable to trigger any [Ca\textsuperscript{2+}]i variations (Supplemental Fig. 3A) or apoptosis in vitro (Supplemental Fig. 3B) in our lymphoma cell lines. Moreover, no potentiation of RTX-induced apoptosis was observed when cells were coincubated with zoledronate in vitro (Supplemental Fig. 3B). However, we confirmed that it significantly reduced calcemia in mice (Supplemental Fig. 3C). Thus, we tested whether

### Table I. Patient and sample characteristics

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F, female; FLIPI, Follicular Lymphoma International Prognostic Index; GCB, germinal center B-cell like; M, male; WHO, World Health Organization.
zoledronate potentiates the antitumoral effect of RTX in vivo. Rag\textsuperscript{2C\textendash/2} mice xenografted with Raji cells were treated three times per week with RTX, alone or in combination with zoledronate. The data obtained showed that cotreatment induced a significant reduction in tumor volume, compared with drug treatment alone (Fig. 4C). In conclusion, these results demonstrated that the Ca\textsuperscript{2+} influx via Orai1-dependent channels repressed the effect of RTX on tumor growth. Consequently, its inhibition by specific inhibitors or hypocalcemic agents, such as zoledronate, may enhance the antitumoral effect of RTX.

**Downstream effectors of RTX-induced Ca\textsuperscript{2+} influx**

These data raised the question of the Ca\textsuperscript{2+}-dependent molecular target contributing to the impairment of RTX-induced apoptosis. Considering that PKCs (28) and, particularly, PKC\textsubscript{b2} (26) have been shown to prevent apoptosis induced by CD95L and that PKC\textsubscript{b} are often upregulated in B cell–derived malignancies (29), we investigated whether PKC\textsubscript{b} acted as a downstream effector of the Ca\textsuperscript{2+} influx mediating inhibition of RTX-induced apoptosis. Pretreatment of SUDHL4 cells with Go6976, an inhibitor of classical PKC isotypes, sensitized cells to RTX-induced apoptosis.

**FIGURE 2.** SOCE inhibition increased rituximab-induced apoptosis. (Aa) SUDHL4 cells expressing shNT or shOrai1 were incubated with RTX (10 \(\mu\)g/ml) for 24 h. Active caspase 8 (left), 9 (middle), and 3 (right) were detected by the Fam-FLICA in vitro caspase detection kit and analyzed by flow cytometry. (Ab) Cells were incubated with RTX (10 \(\mu\)g/ml) in the presence or absence of BTP2 (10 \(\mu\)M) or ML9 (50 \(\mu\)M) for 24 h. Active caspases were detected, as described above. The data represent mean ± SE of three independent experiments.

**FIGURE 3.** Immunostaining showing colocalization of CD95 with Orai1 and STIM1 in CD20 capping in the SUDHL4 cell line. Representative images of (A) colocalization of CD95 with CD20 capping. (B) Colocalization of CD95 with Orai1 and STIM1. Cells were incubated in the presence or absence of RTX (10 \(\mu\)g/ml) at 37°C for 15 min. Cells were fixed and stained with anti-CD95 revealed by donkey anti-mouse Ab coupled to Alexa 594 and anti-CD20 FITC or anti-RTX FITC or anti-STIM1 or anti-Orai1 revealed by donkey anti-rabbit Ab coupled to Alexa 488. Nuclei are depicted in blue. Images were acquired as described in Fig. 1.
Female Rag2/−/− mice were carried out for ML9, three times per week throughout the experiment until the mice were sacrificed. (Fig. 5Ba), whereas PKCB1 and PKCα remained in the cytosol (Fig. 5Bb). This relocation of PKCB2 was completely abrogated in cells pretreated with BTP2 (Fig. 5Ba), suggesting that RTX-induced SOCE activated PKCB2, inducing its distribution into the RTX capping. However, in the Raji cell line, unlike SUDHL4, the PKCβ inhibitor had no effect on RTX-induced apoptosis (Supplemental Fig. 4A) and no relocation of PKCB2 in CD20 capping was observed (Supplemental Fig. 4B), suggesting that the involvement of PKCB2 in the RTX signaling pathway was cell-type specific.

Discussion

In the past decade, a combination of immunotherapy, using anti-CD20 and cytotoxic drugs (like cyclophosphamide, doxorubicine, vincristine, prednisone), has led to marked improvements in treatment response and overall survival in B-NHL. However, treatment resistance and relapse, as well as chemotherapy-related toxicities, remain problematic. Thus, the development of new treatment strategies, including therapeutic Abs and targeted therapy, is particularly challenging. In this context, an intervention in the signaling pathway stimulated by the Ab is assumed to enhance its therapeutic effect. Although rituximab is known to bind to CD20 on lymphoma cells, triggering a cytotoxic effect, the mechanism responsible remains unclear.

Our results revealed that RTX induced colocalization of Orai1, STIM1, CD95, and CD20 in a cluster, eliciting a localized Ca2+ influx, which exerted a negative feedback loop on RTX-induced apoptosis. As this effect was mediated by different downstream effectors, according to the cell line studied, we propose that targeting the RTX-induced Ca2+ influx was likely to improve the RTX response in B-NHL.

We demonstrated that RTX induced a biphasic Ca2+ response, consisting of a rapid, transient peak, followed by a sustained phase. As previously described (6, 8), we confirmed that the peak was due to activation of the IP3 receptors and mobilization of the ER Ca2+ store, whereas the plateau phase consisted of an extracellular Ca2+ influx. Until now, the involvement of CD20 as a calcium channel responsible for Ca2+ influx in response to CD20 ligation was controversial (8–10). Our data revealed that the RTX-induced sustained Ca2+ response originated from the Orai1/STIM1 complex in lipid rafts (10), together with the CRAC channel (30), leading to a rapid, focused, steep Ca2+ increase.

The role of Ca2+ in apoptosis has been extensively investigated (31), but its pro- or antiapoptotic effect seems highly dependent on the stimulus and cell type, as well as the Ca2+ channels and pumps that enable cells to specifically regulate Ca2+-dependent apoptosis. Our experiments demonstrated that inhibition of SOCE by Orai1 down-expression or CRAC inhibitors sensitized B-NHL primary cells and cell lines to RTX-induced apoptosis and enhanced the antitumoral effect of RTX in vivo. Similar results were obtained in vivo with zoledronate, a hypocalcemic agent commonly used in cancer treatment. The enhanced antitumoral effect of RTX in combination with zoledronate is probably due to the hypocalcemic action of zoledronate rather than its direct cellular effect (6, 27), because zoledronate was unable to induce apoptosis or [Ca2+]i.

**FIGURE 4.** SOCE inhibition or hypocalcemic agent increased the antitumoral effect of RTX. (A) Antitumoral effect of RTX increased in mice bearing xenografted tumors of SUDHL4 cells knocked down for Orai1, compared with cells expressing shNT. Female Rag2/−/− mice (n = 8/group) were xenografted s.c. with SUDHL4 cells expressing shRNA or not. Vehicle or RTX (0.01 mg/kg) was administered i.p. three times per week throughout the experiment until the mice were sacrificed. (B) Blockade of Ca2+ influx increased the antitumoral effect of RTX. Female Rag2/−/− mice (n = 8/group) bearing xenografted tumors of SUDHL4 cells were injected i.p. with vehicle, BTP2 (12 μg/kg), ML9 (50 μg/kg), RTX (0.01 mg/kg), or RTX in association with BTP2 or ML9, three times per week throughout the experiment until the mice were sacrificed. (C) Zoledronate enhanced the antitumoral effect of RTX. Female Rag2/−/− mice (n = 8/group) were xenografted s.c. with Raji cells. Mice were injected i.p. three times per week with vehicle, zoledronate (1 mg/kg), RTX (0.01 mg/kg), or RTX plus zoledronate throughout the experiment until the mice were sacrificed. Results are expressed as mean ± SE (*p < 0.05).
variation in our cell lines in vitro. Zoledronate-induced hypocalcemia probably sensitized cells to rituximab-induced apoptosis by decreasing RTX-dependent Ca\(^{2+}\) influx, because we showed that extracellular Ca\(^{2+}\) concentrations modulated the proapoptotic effect of RTX. Thus, our results revealed that Ca\(^{2+}\) influx exerted a negative feedback loop on RTX-induced cell death in B-NHL cells. Similarly, we recently reported that CD95 activation by its ligand CD95L triggered an Orai1-mediated Ca\(^{2+}\) influx, which impaired death-inducing signaling complex formation and caspase 8 activation (26).

Interestingly, the experiments reported in this work revealed that RTX induced relocalization of CD95 in the CD20 capping. The caspase 8 activation and CD95 capping induced by RTX, hallmark of the CD95 activation pathway, confirmed that RTX-induced apoptosis was at least partly due to activation of the CD95 pathway. Previous studies reported crosstalks between RTX and CD95 pathways via the following: 1) the sensitization of B cells to CD95L and TRAIL through upregulation of death-receptor expression induced by RTX (32, 33) and 2) the clustering of CD95 with CD20 in lipid rafts (34).

Several proteins whose activity is controlled by [Ca\(^{2+}\)] increase have been implicated in proapoptotic CD95 signaling (14). The present work revealed that RTX provoked PKC\(\beta_2\) translocation to CD20 capping in a calcium-dependent manner, thus inhibiting apoptosis. It has been reported that members of the PKC family prevent induction of CD95 signaling (28, 35, 36). More recently, in T lymphocytes, it was shown that, on CD95 engagement, Orai1-driven localized Ca\(^{2+}\) influx specifically recruited PKC\(\beta_2\) to the death-inducing signaling complex, thus inactivating the complex and preventing caspase activation and apoptosis (26).

**FIGURE 5.** PKC\(\beta_2\) is a downstream effector of RTX-induced Ca\(^{2+}\) influx in the SUDHL4 cell line. (A) PKC\(\alpha_\beta\) inhibitor sensitized cells to rituximab-induced apoptosis. Cells were incubated with RTX (10 \(\mu\)g/ml) in the presence or absence of Go\(\delta\)6976 (250 nM) and/or BTP2 (10 \(\mu\)M) for 24 h. Active caspase 8 (left), 9 (middle), and 3 (right) were detected by the FAM-FLICA in vitro caspase detection kit and analyzed by flow cytometry. The data represent mean ± SE of three independent experiments (*\(p < 0.05\)). (B) PKC\(\beta_2\) redistributed in CD20 capping. (Ba) SUDHL4 cells were preincubated or not with BTP2 (10 \(\mu\)M) at 37°C for 30 min and then treated with RTX (10 \(\mu\)g/ml) for 15 min. Cells were fixed and stained with anti-CD20 FITC or anti-RTX FITC and anti-PKC\(\beta_2\) revealed by donkey anti-rabbit Ab coupled to Alexa 594. Nuclei are depicted in blue. Images were acquired as described in Fig. 1. (Bb) Cells were incubated in presence or absence of RTX (10 \(\mu\)g/ml) at 37°C for 15 min. Cells were fixed and stained with anti-PKC\(\beta_1\) or anti-PKC\(\alpha\) revealed by donkey anti-rabbit Ab coupled to Alexa 594 and anti-CD20 FITC or anti-RTX FITC. Nuclei are depicted in blue. Images were acquired as described in Fig. 1.
at diagnosis and 90% at relapse (37, 38). Hyperexpression of PKCβ has been associated with resistance to immunotherapy (39) and poor prognosis in DLBCL (38). These observations make PKCβ a putative drug target. To date, several clinical trials testing the impact of Enzastaurin, a PKCβ inhibitor, in aggressive and indolent NHL are ongoing, but, to our knowledge, results are not yet available. Nevertheless, our results suggest that a combination of RTX with a PKCβ inhibitor is not efficient in various NHL because in Raji cells, unlike SUDDL4 cells, PKCβ is not a downstream target of RTX-induced Ca2+ signaling, and further experiments are required to identify this target in this cell line. However, we clearly revealed that inhibition of RTX-induced Ca2+ influx sensitizes all NHL cells tested in this study to RTX, suggesting that the targeting of the Ca2+ influx is a likely strategy for improving anti-CD20 immunotherapy in a large number of patients.

To date, intense drug discovery efforts have focused on developing small-molecule CRAC-channel antagonists (40–42) or specific Orai1 Abs (43) to treat autoimmune diseases and chronic inflammation. We propose using these compounds in association with RTX or, more generally, with drugs inducing a CD95-dependent apoptotic pathway, to constitute a new therapeutic opportunity for patients.

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