Enhancement of Rituximab-induced cell death by the physical association of CD20 with CD40 molecules on the cell surface

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Abstract

CD20 is an attractive therapeutic target given the success of its monoclonal antibody, Rituximab, in the treatment of B-cell malignancies and B-cell-mediated autoimmune diseases. Treatment with Rituximab causes a rapid depletion of B cells and a decrease in disease symptoms. Despite the clinical efficiency of Rituximab, its mechanism of action is not completely understood. In this study, we aimed at further investigating the Rituximab-induced cell death and the factors affecting such responses. Our results indicate that Rituximab-induced cell death depends on the nature of the cells and levels of CD20 expression on the cell surface. Coexpression of CD20 with CD40, a member of the TNF receptor family that is known to be physically associated with CD20 on the cell surface, enhances the apoptotic response induced by Rituximab. Inhibiting the formation of CD40 disulfide-bound-homodimers, a process required for some CD40 signaling, further enhances Rituximab-induced cell death. Cell death induced by anti-CD40 mAb is also upregulated by the presence of CD20, suggesting a bidirectional influence of the CD20/CD40 association. Moreover, treating cells with both anti-CD20 and anti-CD40 antibodies improves the cell death response induced by a single-agent treatment. These results highlight the role of the CD20/CD40 association in triggering B-cell depletion and may pave the way for an alternative more efficient therapeutic strategy in treating B-cell-mediated disorders.

Keywords: association, CD20, CD40, cell death, Rituximab

Introduction

CD20 is a non-glycosylated, tetraspanning B-cell membrane protein with a molecular weight of 33–37 kDa that is expressed from the pre-B to mature B-cell stage but is lost in plasma cells (1, 2). Our understanding of CD20 signaling events comes from the use of specific mAbs. Upon its engagement with a specific mAb, CD20 translocates into the lipid raft fraction where it initiates a number of signaling pathways, including src-tyrosine kinases (Lyn, Fyn, Lck) (3). CD20 was shown to associate with the BCR and trigger calcium flux in B cells by activating signaling pathways similarly activated by the latter (4, 5). Furthermore, the activation of CD20 leads to phospholipase C-γ phosphorylation and mitogen-activated protein kinase (MAPK) pathway activation (6). This gives rise to the cleavage of phosphatidylinositol trisphosphate, which generates inositol trisphosphate and diacylglycerol resulting in the activation of protein kinase C (3, 7–10).

The most important observation that has been made with respect to CD20 function is the finding that treatment with anti-CD20 mAb triggers a rapid B-cell depletion (11–13). Since the approval of Rituximab by the Food and Drug Administration to treat refractory or relapsed indolent B-cell non-Hodgkin’s lymphoma, its usage has revolutionized the treatment of B-cell malignancies (14). Recently, Rituximab has been used in the treatment of rheumatoid arthritis (RA) patients, where a significant decrease in arthritic symptoms was observed (15, 16). While Rituximab can induce death in pre-B cells to pre-plasma cells, the germinal center is resistant to the cytotoxicity of Rituximab, mainly due to local protective mechanisms (17). The efficiency of Rituximab is in part due to its chimeric structure, where the
CD20-binding Fab fragment is murine and the Fc portion is human (18). Rituximab can deplete B cells in three ways (1): antibody-dependent cell-mediated cytoxicity (ADCC) (2), complement-dependent cytoxicity (CDC) (3) and induction of cell death (19). Although the first two pathways involved in B-cell depletion are now well established, the direct cell death is not yet completely understood.

The Fc portion of Rituximab is responsible for the ADCC and the CDC pathways, stimulating effector cells via their Fc receptor, and activating the classic complement pathway via binding to C1q, respectively (20). The presence of the human Fc portion prolongs the half-life of Rituximab, which seems to be important for its therapeutic potency (21). The Fc portion can also bind to C1q, activating the classic complement pathway and releasing anaphylatoxins (C3a, C4a and C5a) and opsonins such as C3b (11). As to the role of Rituximab in cell death, some caspase-independent pathways have been postulated, but the apoptotic pathway is thought to involve three main caspase-dependent mechanisms: the activation of src-tyrosine kinases (Lyn, Fyn and Lck) (22), the activation of Fas signaling (23) and the inhibition of major survival pathways such as p38 and ERK1/2 MAPKs, nuclear factor κB and AKT (24, 25).

The ability of Rituximab to activate many signaling pathways leading to cell death and the lack of cell death domain in the cytoplasmic tail of CD20 strongly suggest the contribution of other surface molecules in the Rituximab-induced response. Upon its ligation with specific mAb, CD20 was shown to physically and functionally associate with the fibroblast growth factor receptor 3 (FGFR3), whereby an inhibition of the tyrosine kinase activity of the FGFR3 downregulated the Rituximab-mediated proliferation inhibition (26). Along the same line of evidence, we previously reported that CD20 is physically and functionally associated with CD40, a member of the TNF receptor family (27). CD40 is expressed in a large variety of cells including B cells and is often used as a marker of all stages of B-cell differentiation (28). In addition to its established role in cellular activation (28, 29), CD40 was shown to play a direct role in the induction of specific cell death that is caspase and protein synthesis independent (30). Thus, data demonstrating an association of CD40 and CD20 on the surface of B cells, and those identifying a direct function of CD40 in inducing specific cell death, could suggest a possible contribution of CD40 to the apoptotic response mediated by the Rituximab/CD20 complex.

In this study, we further investigate the mechanisms and the influence of CD20/CD40 association on the Rituximab-induced cell death response, as well as on CD40-mediated apoptotic events. While confirming the physical association between CD20 and CD40 on the cell surface, our results show that such association enhances the cell death response mediated by either CD20 or CD40. Interestingly, our data demonstrate that inhibiting CD40 disulfide-bound (db)-homodimer formation further upregulates CD20-induced cell death. Moreover, treating cells with both anti-CD40 and anti-CD20 mAbs induced an additive cell death response. Our data delineate an important mechanism of the Rituximab/CD20-mediated cell death response while expanding our knowledge of B-cell depleting therapies and their efficiencies in the treatment of B-cell-mediated diseases.

Methods

Reagents and antibodies

Hybridomas producing mAbs directed against the intracellular domain of HLA-DR α chain (DA6-147: IgG1), the extracellular domain of HLA-DR (L243: IgG2a), CD20 (1F5: IgG2a) and CD40 (G28-5: IgG1) were purchased from ATCC (Manassas, VA, USA). The 8C12 control mAb (IgG2a) was produced in our laboratory. The mAbs secreted by these hybridomas were purified on Protein G/A Sepharose columns (Sigma–Aldrich, Oakville, ON, Canada). Rituximab, a chimeric anti-human CD20 mAb, was a generous gift of Dr G. Hoffman. The human IgG (IgG) used as a negative control, the Affinity-Pure F(ab′)2 fragment goat anti-human IgG, and HRP-conjugated goat anti-rabbit IgG (H+L) and HRP-conjugated goat anti-mouse IgG (H+L) secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The Alexa-488 goat-conjugated anti-mouse IgG was from Invitrogen Life Technology (Burlington, ON, Canada). Propidium iodide (PI; Invitrogen) was used to quantify cell death by flow cytometry.

Cells

The Burkitt’s lymphoma BJAB and Ramos cell lines (ATCC, Rockville, MD, USA) were maintained in RPMI supplemented with 5% of heat inactivated fetal bovine serum (FBS). HEK-293 cells (ATCC) transfected with various plasmids were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin/glutamine (PSG, Life Technologies, Gibson/BRL, Burlington, ON, Canada) and 1600 µg ml⁻¹ of neomycin (InvivoGen, Cedarlane Laboratories, Burlington, ON, Canada) or 800 µg ml⁻¹ of hygromycin B (Wisent, Montreal, QC, Canada). Peripheral blood mononuclear cells were isolated by Ficoll gradient density centrifugation, and B cells were purified by negative selection with antibody-coated magnetic beads in a Robosep instrument (Stem Cell Technology).

Mutagenesis and cell transfections

PCR-directed mutagenesis was used to construct wild-type (WT) CD40 (hCD40WT) and CD20 (hCD20WT) and their mutants. The CD20 and CD40 mutants were PCR-amplified using oligonucleotide primers containing appropriately positioned point mutations and BCMGShCD20.Neo and pcDNA3.1hCD40.Hyg, respectively, as templates. The PCR products were inserted into their respective plasmids. DNA sequencing confirmed that all PCR products were free of undesired mutations. Transfections were performed by calcium phosphate precipitation. Prior to transfection, 1 x 10⁶ HEK-293 cells were seeded in 10-cm culture plates and were incubated for 24 h. Stably infected cells were selected in the presence of hygromycin and/or neomycin. HEK-293 cells stably expressing human WT CD20 (hCD20WT) and cells expressing different levels of human CD20 (hCD20C1, hCD20C2, hCD20C3, hCD20C4) were generated. In addition, HEK-293 cells were
double transfected with human CD20WT and CD40WT (hCD20/CD40WT), with human CD20WT and CD40 truncated mutant (hCD20/CD40Δ), with human CD20WT and CD40C238A mutant (hCD20/CD40C238A) or, as used controls, with human CD20WT and pcDNA3.1 empty vector (hCD20/pcDNA3.1), or with CD40WT and BCMGS empty vector (hCD40/BCMGS).

Flow cytometry analysis

HEK-293/hCD20WT cells expressing different levels of human WT CD20 (hCD20-C1, hCD20-C2, hCD20-C3, hCD20-C4) were harvested and were incubated with intracellular HLA-DR α chain mAb DA6-147 (1 μg per 100 μl), anti-HLA-DR mAb L243 (1 μg per 100 μl) or anti-CD20 mAb 1F5 (1 μg per 100 μl) for 30 min on ice. The cells were washed with ice-cold PBS/2% FBS, and viable cells were incubated with Alexa fluor-488-conjugated anti-CD20/CD40-C238A were also treated with the same antibodies. The human B cells were incubated with anti-CD20 mAb 1F5 (IgG2a; 1 μg/ml−1) used alone or combined with G28-5 (0.25 μg/ml−1 for the HEK-293/hCD20 cells and 10 μg/ml−1 for the BJAB cells) was added. No Rituximab was included in the control wells. The plates were incubated for 16 h at 37°C. Cells were collected and were incubated with 1 μg/ml−1 of PI for 10 min on ice prior to being gated by FACScan.

Immunoprecipitation and western blotting

HEK-293 cells expressing hCD20/CD40WT or hCD20/CD40-Δ (5 × 10⁶ cells per ml) were lysed for 1 h on ice in 400 μl of lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% CHAPS) supplemented with protease inhibitors (Invitrogen). The lysates were sonicated three times for 3 s at 25% amplification and were then centrifuged at 9,300 x g for 15 min at 4°C. Dynabeads (DynMag-Spin, Invitrogen, Norway) were prepared according to the manufacturer’s instructions. The lysates were centrifuged and 800 μl of the lysate supernatants were added to 200 μl of Dynabeads conjugated with 10 μg of antibody (DA6-147, hIgG, G28-5 or Rituximab). The mixtures were incubated for 16 h with rotation at 4°C. The bead-conjugated antibodies were then placed on a micro-column and a magnet was used to separate the beads from the supernatants. The supernatants were discarded, and the beads were rinsed five times with 200 μl of lysis buffer and were resuspended in 40 μl of lysis buffer. Preheated charging buffer (10 μl; 50 mM Tris HCl, 50 mM dithiothreitol, 5% SDS, 0.005% bromophenol blue, 10% glycerol) was added, and the beads were incubated at 95°C for 5 min. Proteins were separated by electrophoresis on 10% acrylamide gels and were transferred to polyvinylidene difluoride membranes. The membranes were washed with 5% milk/TBS-T for 30 min at room temperature and were first incubated with HRP-conjugated goat anti-rabbit IgG (GAR-HRP, 1:50,000) for 1 h at room temperature. The membranes were washed 3 times with TBS 0.1% Tween 20 for a total of 15 min and were then incubated with an HRP substrate (Western Lightning Plus ECL, PerkinElmer, Waltham, MA, USA). After washing three times with Tris-buffered saline (TBS) 0.1% Tween 20 for a total of 15 min, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG1 antibody (GAR-HRP, 1:50,000) for 1 h at room temperature. The membranes were washed 3 times with TBS 0.1% Tween 20 for a total of 15 min and were then incubated with an HRP substrate (Western Lightning Plus ECL, PerkinElmer, Waltham, MA, USA) in accordance with the manufacturer’s instructions to reveal the protein bands.

Results

Rituximab-induced cell death via CD20 is cell line dependent

While Rituximab was capable of inducing cell death in some B cell lines such as the Burkitt lymphoma cell lines, Raji, and...
BJAB (26, 31), in others such as Ramos Burkitt lymphoma cells, results were inconsistent. Thus, in the first set of experiments, we aimed at evaluating the susceptibility of different B cell lines, and resting B cells, to Rituximab-induced cell death. Cells were incubated with different concentrations of Rituximab overnight and cell death was evaluated. Although Rituximab did not induce any detectable response in Ramos cells (Fig. 1A), it was capable of inducing significant cell death in BJAB, resting B cells and other B cell lines (Raji and LG2, data not shown) in a dose-dependent manner. Cell death in BJAB was induced at 1 µg ml⁻¹ of Rituximab and reached 40% in the presence of 10 µg ml⁻¹. In resting human B cells, our results demonstrated 22% cell death after Rituximab treatment. The failure of Rituximab to induce such a response in Ramos cells can be due to the levels of CD20 on cell surface, the maturation status of the cell or both. To verify the first hypothesis, we analyzed the expression of CD20 by flow cytometry. Ramos cells expressed less CD20 than BJAB on their surface but higher than resting B cells (Fig. 1B), suggesting that Ramos cells exhibit a resistance to Rituximab-induced cell death in spite of levels of CD20 on the cell surface. As to the maturation status of the cells described.

**Fig. 1.** Rituximab/CD20 induced cell death in human B cells and in some but not all B cell lines. (A) Ramos, BJAB and purified B cells (5 × 10⁶ cells per ml) were treated with different concentrations of Rituximab (RTX; 1; 5; 10 µg ml⁻¹) or hIgG (as negative control). Cell death was quantified by PI uptake and flow cytometry. The results are presented as means ± SD (n = 3), ***P < 0.001. (B) Cells were first incubated with 1F5 anti-CD20, followed by Alexa-488-conjugated goat anti-mouse IgG. IgG2a antibody was used as an isotype-match control. CD20 expression was assessed by flow cytometry. Data are representative of three independent experiments.
above, Ramos B cells are immature cells, while BJAB and human B cells are mature ones. Thus, our data demonstrating a cell-depleting role of Rituximab in the latter two cell types, and its absence in Ramos cells, suggest that the maturation stage of B cells might influence their Rituximab-induced cell death response.

**PMA-priming or cross-linking with a secondary antibody sensitizes Ramos cells to Rituximab-induced cell death**

Ramos B cells have been previously shown to exhibit a decreased sensitivity to cell death mediated by Rituximab treatment (32, 33). Given the inability of Rituximab to induce death in Ramos B cells in our system, we wanted to assess the possibility of boosting such a response. In the first set of experiments, we aimed at investigating if the activation state of Ramos B cells could affect the cell death response induced by the Rituximab/CD20 complex. For this purpose, Ramos cells were left untreated or treated with PMA (1 ng ml⁻¹) for 24h, followed by Rituximab for an additional 16h. Our results show that treating Ramos B cells with PMA upregulated the expression of CD20 on the cell surface (Fig. 2A) and sensitizes Ramos cells to Rituximab-induced cell death (death rate of 27% in PMA-activated the cells) (Fig. 2B). In a second set of experiments, we wanted to assess the possibility of boosting the cell death response in Ramos cells by cross-linking Rituximab with a secondary antibody and clustering the molecule on cell surface. We pretreated Ramos cells with 10 μg ml⁻¹ of Rituximab for 30min, followed by an overnight incubation with 10 μg ml⁻¹ of a secondary antibody, a goat anti-human IgG antibody that cross-links Rituximab with its F(ab')2 fraction. Our results show a 37% cell death response in Ramos cells treated with Rituximab in the presence of the secondary antibody (Fig. 2C). Thus, our data suggest that Ramos B cells, which exhibit a decreased susceptibility to Rituximab-induced cell death, require a boosting step such as cross-linking with a secondary antibody or priming with PMA for a proper cell death response.

**Rituximab-induced cell death is dependent on the level of CD20 expression**

To confirm that the level of CD20 on the cell surface is a factor affecting the success rate of Rituximab in cell depletion, as an increased expression of the molecule allows an intense recruitment of the antibody onto the plasma membrane (19), several attempts were made to sort tonsil and peripheral blood resting B cells expressing various levels of CD20 but unfortunately failed. To overcome this problem, we transfected HEK-293 cells with human CD20. In a first set of experiments, HEK-293/hCD20WT cells were incubated overnight with different concentrations of Rituximab (0.2, 0.4, 0.8, or 1.6 μg ml⁻¹), or with hIgG used as a negative control. Results demonstrated a dose-dependent cell death response following stimulation with Rituximab (Fig. 3). The cell death induced in HEK-293/hCD20 cells began at 0.2 μg ml⁻¹ of Rituximab and was increased to 46.9% in the presence of 1.6 μg ml⁻¹ of Rituximab. Next, HEK-293 cells ectopically stably expressing various levels of CD20 were sorted and named clone 1 (C1), C2, C3 and C4 (Fig. 4A). Upon stimulation of these cells with Rituximab, the cell death response was shown to increase depending on the level of CD20 expression on the cell surface (Fig. 4B). HEK-293/hCD20 C1 failed to exhibit any cell death, while C3 and C4 showed a significant death response following Rituximab treatment (death rate of 47 and 61.2%, respectively). These data confirm that the level of expression of CD20 on the cell surface ensures a successful cell death response upon Rituximab treatment.

**Rituximab-induced cell death is Fc receptor independent**

Thus, in our system, since HEK-293 and BJAB cells express Fc receptors, we wanted to determine whether Rituximab-induced cell death via CD20 was dependent on Fc receptor binding. BJAB and HEK-293/hCD20 cells were pretreated with different concentrations of hIgG to block the Fc receptors expressed on the cell surface. Cells were then stimulated with optimal Rituximab concentrations (Fig. 5A and B). Blocking the Fc receptors did not alter the cell death response triggered in BJAB and HEK-293/hCD20 cells. These results indicate that Rituximab-induced cell death via CD20 is independent of Fc receptors and could be mediated by the activation of apoptotic events. Comparable results were obtained when F(ab')2 fragments of Rituximab were used (data not shown).

**Physical association of CD20 with CD40 enhances Rituximab-induced cell death**

We have previously reported that CD20 is physically and functionally associated with CD40 on human B cells (27). In addition, several groups including ours have shown that the engagement of CD40 is capable of inducing apoptosis in B cell lines and malignant cells (30, 34, 35). Thus, in this study, we investigated the influence of such an association on the cell death response induced by Rituximab. For this purpose, HEK-293 cells stably expressing CD20 (clone 3) were cotransfected with empty vector (HEK-293/hCD20/pcDNA3.1), CD40WT (HEK-293/hCD20/CD40WT) or truncated CD40 lacking its cytoplasmic domain (HEK-293/hCD20/CD40Δ). Cells expressing comparable levels of CD20 and CD40 were sorted and used in the subsequent experiments (Fig. 6A). HEK-293/hCD20/pcDNA3.1, HEK-293/hCD20/CD40WT and HEK-293/hCD20/CD40Δ cells were stimulated with different concentrations of Rituximab. HEK-293/hCD20/CD40WT cells exhibited a more pronounced cell death response to 0.8 μg ml⁻¹ Rituximab, compared with cells expressing only CD20 (64 and 48.8%, respectively, Fig. 6B). Interestingly, HEK-293/ hCD20/CD40Δ cells exhibited a 47.4% death rate in response to the highest concentration of Rituximab used (0.8 μg ml⁻¹), similarly to HEK-293/hCD20/pcDNA3.1 cells (48.8%) and 16.6% less than that of HEK-293/hCD20/CD40WT cells (Fig. 6B). These results confirm that CD20 is constitutively associated with CD40 and demonstrate that such an association increases the efficiency of Rituximab/CD20-induced cell death. Indeed, the enhancing role of CD40 toward the cell death response induced by the Rituximab/CD20 complex involves the cytoplasmic domain of CD40.

Next, we aimed at confirming the association of CD20 with CD40 in these cotransfected cells and assessing the implication of the cytoplasmic domain of CD40 in the physical association of CD20 with CD40. The above described cells were
Fig. 2. PMA-priming and cross-linking Rituximab with a secondary antibody sensitizes Ramos cells to cell death. (A and B) Activating Ramos B cells with PMA. Ramos cells (0.5 × 10^6 cells per ml) were incubated with 1 ng ml⁻¹ of PMA for 24 h at 37°C, or left non-treated (NT). (A) CD20 surface expression was assessed by flow cytometry. (B) For cell death assessment, PMA-activated cells were stimulated with Rituximab for 16-h incubation at 37°C, or with hIgG (as negative control) and cell death was quantified by PI uptake and flow cytometry. (C) Cross-linking Rituximab with a secondary antibody. Ramos cells (5 × 10^6 cells per ml) were pretreated with 10 µg ml⁻¹ of Rituximab (RTX) or hIgG (as negative control) for 30 min at 37°C. The cells were washed, plated and incubated with 10 µg ml⁻¹ of a goat anti-human IgG (GAH) secondary antibody. Cell death was quantified by PI uptake and flow cytometry. Results are presented as means ± SD (n = 3), ***P < 0.001.
lysed and immunoprecipitated with anti-CD40 mAb (G28-5), Rituximab or isotype control antibody. Results show that even when the cytoplasmic domain of CD40 is absent, the anti-CD40 antibody is capable of coprecipitating CD40 with CD20 (Fig. 6C), suggesting that the association of CD20 with CD40 is independent of the cytoplasmic domain of the latter.

Inhibiting CD40 db-homodimer formation enhanced cell death induced by anti-CD20

We have reported that oligomerization of CD40 with trimeric CD40 ligand or cross-linked antibodies leads to CD40 db-homodimer formation, an event that is required for some CD40-induced signaling (36). More recently, we have demonstrated that substituting cysteine 238 of the cytoplasmic tail by alanine (A) abolished CD40 db-homodimer formation (37) and enhanced the apoptotic role of CD40 (30). Here, we wanted to determine the role of CD40 db-homodimers in cell death induced by Rituximab. For this purpose, HEK-293 cells co-expressing hCD20 and hCD40WT (HEK-293/hCD20/CD40WT) or hCD40 containing a mutation converting cysteine 238 to alanine (HEK-293/hCD20/CD40-C238A) were used. A similar level of expression of CD20 was exhibited in HEK-293/hCD20/pcDNA3.1, HEK-293/hCD20/CD40WT and HEK-293/hCD20/CD40-C238A, and a similar level of CD40 was expressed by the latter two cell line groups (Fig. 7A).

Upon stimulation with different concentrations of Rituximab, HEK-293/hCD20/CD40-C238A cells exhibited an increased cell death response at a rate of 65% compared with 59% in cells expressing CD20 and CD40WT (Fig. 7B). These data indicate that inhibiting CD40 db-homodimer formation influences the enhancing role of CD40 toward CD20-induced cell death, just like it does for the cell death triggered by CD40 stimulation.

Association of CD20 with CD40 enhances cell death induced by CD40

Given our current data outlining the enhancing role of CD40 toward CD20-induced cell death, our next question was...
does the CD20/CD40 dyad affect the cell death response induced by CD40 itself? For this purpose, we stimulated HEK-293/hCD20/CD40WT cells, and cells expressing only CD40 (HEK-293/hCD40/BCMGS) as a control, with different concentrations of G28-5 (Fig. 8). G28-5-mediated cell death was significantly increased in HEK-293 cells expressing both CD20 and CD40 at a rate of 60.8% compared with 30.1% in HEK-293/hCD40 cells. These results suggest that the impact of the CD20/CD40 association might be bidirectional, influencing signaling via both molecules.

Combination of anti-CD40 and Rituximab leads to an additive cell death response

Given our data above indicating that CD20/CD40 association positively influences the cell death signal induced via
Results are presented as means ± SD at 37°C, cell death was quantified by PI uptake and flow cytometry. BJAB cells), or left non-stimulated (NS). Following a 16-h incubation μ of Rituximab and 0.25 μ treated cells mentioned above with different concentrations (Fig. 9A). A similar additive effect was obtained when we stimulated with increasing concentrations of G28-5 alone cells exhibited increased cell death, compared with cells G28-5 (increasing concentrations) and Rituximab (0.2 μCD40WT or BJAB cells were treated with a combination of antibodies. When HEK-293/hCD20/CD40WT or BJAB cells were treated with a combination of G28-5 (increasing concentrations) and Rituximab (0.2 μg ml⁻¹ for HEK-293/hCD20/CD40WT and 1 μg ml⁻¹ for BJAB), cells exhibited increased cell death, compared with cells stimulated with increasing concentrations of G28-5 alone (Fig. 9A). A similar additive effect was obtained when we treated cells mentioned above with different concentrations of Rituximab and 0.25 μg ml⁻¹ of G28-5 (Fig. 9B). Thus, the treatment with a combination of Rituximab and G28-5 improved the cell death response induced by a single-agent treatment in HEK-293 cells expressing both CD20 and CD40, as well as in BJAB cells. These data suggest that the usage of a dual antibody treatment with anti-CD20 and anti-CD40 might increase the efficiency of the Rituximab treatment in depleting B cells.

CD20, a B-cell-specific marker, has gained great interest in the field of therapeutic strategies in B-cell malignancies and many B-cell-mediated autoimmune diseases, owing to the fact that CD20 is the target of a clinically efficient chimeric mAb, Rituximab. In this study, we investigated the mechanisms mediating the cell death response induced by Rituximab and the role of CD20/CD40 association in such a response. Our results show that the susceptibility of B cells to Rituximab-induced cell death is different among B cell lines. The death response mediated by Rituximab is dependent on the level of CD20 expression on the cell surface and independent of the Fc fragment. We also showed that CD20 associates with CD40 on the cell surface, a property that upregulates both CD20- and CD40-mediated cell death responses upon stimulation with their respective antibodies. In addition, our data demonstrated an additive effect of both anti-CD20 and anti-CD40 antibodies in inducing cell death.

B cell lines [BJAB, Raji and LG2 (data not shown)], resting B cells and HEK-293 cells (ectopically expressing CD20) exhibited a significant death response upon their stimulation with Rituximab. However, the Ramos B cell line was shown to be insensitive to Rituximab-induced cell death. The resistance of this cell line to cell death was previously described by Vega et al. (33). The authors, using several B cell lines including Ramos, reported that while Rituximab or a Fas ligand agonist antibody failed to induce B-cell death when used alone, they were capable of provoking apoptotic signals if used in combination. This finding and ours suggest that the Ramos B cell line might be more resistant to Rituximab-induced apoptosis and require an enhancement such as an extensive cross-link of the Rituximab. Indeed, our Ramos B cells exhibited a Rituximab-induced death response upon cross-linking Rituximab with a secondary antibody. However, we should be noting herein the discrepancy of our result with other studies that previously demonstrated that Rituximab by itself was capable of inducing apoptosis in Ramos B cells in the absence of clustering with a secondary antibody (38). The disagreement between these previous results and more recent ones, including ours, could relate to different incubation conditions and/or culturing media that plays a key role in Ramos homotypic cell to cell adhesion. Another process increasing the sensitivity of Ramos B cells to Rituximab was the induction of cell activation and the subsequent upregulation of CD20 expression on the cell surface. Indeed, the elevated expression levels of CD20 on the B-cell surface is one of the reasons making this molecule a good target for cell-depleting antibody-based attack (19, 39–41). Our data confirm this finding whereby our HEK-293 cells ectopically expressing an increased level of CD20 exhibited a higher susceptibility to Rituximab-induced cell death, compared with cells with less surface expression of CD20.

Rituximab can induce B-cell depletion in three ways (1): ADCC (2), CDC and (3) induction of cell death (19). The ADCC and CDC modes of action of Rituximab are Fc-dependent mechanisms. Our results demonstrated the Rituximab-induced cell death was not mediated by the Fc fraction of the Ab, reflecting a direct effect on the cell itself leading to cell death or apoptosis. Indeed, many apoptotic
Fig. 6. The physical association of CD20 with CD40 enhances cell death via CD20. HEK-293 cells were stably transfected with CD20, CD40 or a mutated CD40 lacking the cytoplasmic domain (CD40Δ). (A) CD20 and CD40 expressions were assessed by flow cytometry, using anti-CD20 (1F5), anti-CD40 (G28-5) or isotype control (IC). (B) HEK-293/hCD20/pcDNA3.1, HEK-293/hCD20/CD40WT cells and HEK-293/hCD20/CD40-Δ cells were incubated for 16 h at 37°C with different concentrations of Rituximab (RTX; 0.2; 0.4; 0.8 µg ml⁻¹), or left non-stimulated (NS). Cell death was quantified by PI uptake and flow cytometry. The results are expressed as means ± SD (n = 3), ***P < 0.001. (C) HEK-293/hCD20/CD40WT and HEK-293/hCD20/CD40-Δ cells were lysed using a CHAPS lysis solution. Each cell type (5 × 10⁶ cells) was added to the corresponding magnetic protein G beads coated with 10 µg ml⁻¹ of G28-5 or 10 µg ml⁻¹ of RTX and with DA6-147 or hIgG used as negative controls, respectively. Immunoblotting was performed using anti-CD20, GST-77 and anti-CD40, H120 antibodies.
signals have been associated with CD20 stimulation, including phosphorylation of protein tyrosine kinases, Lyn and Lck, upregulation of intracellular Ca\(^{2+}\) levels, inhibition of the Raf-MEK1/2-ERK1/2 signaling pathway, downregulation of the oncoprotein Bcl-xL, transcription repressor yin-yang-1, and activation of caspases such as caspase-3 (33, 38, 42, 43).

Even though beyond the scope of our study, which focuses on another aspect of the role of CD20 in cell death, namely its association with CD40, it would be interesting to identify in future studies the intracellular modulators involved in the apoptotic effect of CD20/Rituximab toward B cells.

Interestingly, CD20 was shown to associate with some biologically active molecules on the surface of B cells. Upon its engagement with Rituximab (type I anti-CD20 mAbs), CD20 colocalized with the BCR and used similar intracellular mechanisms, in a process termed ‘hijacking’ to induce a Ca\(^{2+}\) increase leading possibly to apoptosis (5). More recently, a physical and functional association of CD20 with FGFR3 was demonstrated in the Burkitt lymphoma cell line Raji and BJAB cells upon their treatment with the anti-CD20 antibody, Rituximab. Inhibiting the tyrosine kinase activity of the FGFR3 downregulated the Rituximab-mediated growth arrest (26). Another molecule shown to be associated with CD20 is the TNF receptor family member, CD40. Indeed, CD40 was demonstrated to induce its internalization and that of CD20 into cytoplasmic vesicles in human B cells (44). Interestingly, we previously reported the association of CD20 with MHC class II and CD40 on the surface of human B cells.

![Fig. 7. Inhibiting CD40 homodimer formation upregulates cell death induced by CD20. HEK-293 cells were stably cotransfected with CD20 and the mutated form of CD40 (CD40C238A) unable to form the disulfide-bond homodimer. (A) The expression of CD20, CD40 and CD40C238A was assessed by flow cytometry using anti-CD20 (1F5), anti-CD40 (G28-5) or isotype control (IC). The mean fluorescent intensity is represented in the flow cytometry graph. (B) HEK-293/hCD20/CD40WT and HEK-293hCD20/CD40C238A were incubated for 16 h at 37°C with different concentrations of Rituximab (RTX; 0.2; 0.4; 0.8 µg ml\(^{-1}\)), or left non-stimulated (NS). HEK-293/hCD20/pcDNA3.1 cells were used as control. The cell death was measured by 1 µg ml\(^{-1}\) of PI and flow cytometry. The results are expressed as means ± SD (n = 3), **P < 0.01; ***P < 0.001.

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Such association was biologically active in terms of mediating B-cell homotypic adhesion. Ligating CD20 with mAb R21 blocked the lymphocyte function antigen (LFA)-1-independent but not the LFA-1-dependent homotypic adhesion of B cells induced via MHC class II or CD40, suggesting a functional cross talk between the latter surface molecules and their associated CD20 (27). Other biological responses such as cell death could be mediated by the association of CD20 with CD40. Indeed, CD40 was shown to sensitize cells derived from chronic lymphoblastic leukemia patients to the cytotoxic effects of Rituximab (45). These results are concomitant with the previously reported role of CD40 in inducing apoptosis. Interestingly, CD40 was demonstrated to induce cell death via a lysosomal membrane permeabilization pathway triggering the release of cathepsin B in the cytosol (30). In the aim of investigating the role of the CD20/CD40 association in CD20-induced cell death, our current data first confirm the physical association between both molecules and then demonstrate that cells transfected with both CD20 and CD40 exhibited an increased cell death in response to Rituximab, compared with cells transfected with only CD20. Thus, the association with CD40 may be an additional mechanism by which the Rituximab/CD20 complex is inducing specific death in B cells. Interestingly, we also demonstrated that the presence of CD20 on the surface of cells upregulated the cell death response induced by CD40 itself upon its ligation with a specific antibody. These data further outline the efficiency of the CD20/CD40 association as a biologically active mechanism by itself, capable of influencing cellular responses and affecting biological outcomes.

The enhancing role of CD40 toward the CD20-mediated cell death is shown to be dependent upon the cytoplasmic domain of CD40, while the physical association itself did not involve the same domain. The involvement of the CD40 cytoplasmic domain in the functional cross talk between CD40 and CD20 is in concordance with its well-documented role in CD40-mediated signaling (28, 30). The CD40 db-homodimer formation mediated by the residue C238 is another factor affecting CD40-induced cell death and could be at play in the enhancing role of CD40 toward the apoptotic response induced by the

![Fig. 8. The physical association of CD20 with CD40 enhances cell death via CD40. HEK-293 cells were stably transfected with CD20 or CD40. (A) CD20 and CD40 surface expression was assessed by flow cytometry using anti-CD20 (1F5), anti-CD40 (G28-5) or isotype control (IC). (B) HEK-293/hCD40/BCMGS and HEK-293/hCD20/CD40WT were incubated with different concentrations of G28-5 (0.25; 0.5, 1 μg ml⁻¹) or with DA6-147 (1 μg ml⁻¹) as a negative control, for 16 h at 37°C. Cell death was quantified by PI (1 μg ml⁻¹) and analyzed by flow cytometry. The results are expressed as means ± SD (n = 3), ***P < 0.001.](https://academic.oup.com/intimm/article-abstract/26/8/451/2950783)
Fig. 9. A combination of anti-CD20 and anti-CD40 antibodies enhances the cell death response. HEK-293 cells stably transfected with CD20 and CD40 and BJAB cells were used. (A) Cells were stimulated with different concentrations of G28-5 incubated alone or combined with one concentration of Rituximab (RTX; 0.2 μg ml−1 for HEK-293/hCD20/CD40WT and 1 μg ml−1 for BJAB cells), and DA6-147 was used as a negative control. (B) Cells were stimulated with different concentrations of RTX incubated alone or combined with one concentration of G28-5 (0.25 μg ml−1), and hlgG was used as a negative control. Cell death was quantified by PI (1 μg ml−1) and analyzed by flow cytometry. The percentage of specific cell death was obtained by subtracting the corresponding isotype control. The results are expressed as means ± SD (n = 3), **P < 0.001.
Conventional chemotherapy or a disease-modifying antirheumatic drug was obtained when Rituximab treatment was combined with the Canadian Arthritis Network (CAN60594).

Rituximab has been long proven to efficiently lead to B-cell depletion, particularly in lymphomas and autoimmune conditions such as RA (14, 16, 47). However, longer term efficiency was obtained when Rituximab treatment was combined with conventional chemotherapy or a disease-modifying antirheumatic drug in the case of RA (48, 49). As to CD40, its high expression on B cells, monocytes and macrophages in synovial fluids of RA patients (28), in addition to its implication in apoptotic and antiapoptotic events in lymphoma B cells (50, 51), makes it an interesting target for the treatment of these diseases. Interestingly, in our study, treating cells with both anti-CD20 (Rituximab) and anti-CD40 (G28-5) antibodies leads to an additive cell death, compared with stimulation with a single antibody. Our results are in accordance with data reported by Lewis et al. (52) in 2011, whereby simultaneously targeting CD40 with the humanized anti-CD40 mAb, Dacetuzumab and CD20 with Rituximab significantly improves the in vivo activity of Rituximab. They suggested that these mAbs have distinct but complementary mechanisms of action against tumor cells. Thus, our data combined with the above findings, in addition to confirming the importance of CD20/CD40 association in B-cell death, identify potentially new therapeutic strategies for a more efficient and longer lasting use of the Rituximab regime in the treatment of B-cell-mediated diseases.

In summary, targeting CD20 in B-cell-depleting therapies yielded successful outcomes. However, in spite of the clinical success of anti-CD20 regimens including mAbs such as Rituximab, a clear idea of their mechanisms of action remains to be elucidated. Our data characterized the Rituximab-induced cell death, more specifically in terms of the role of CD20/CD40 association in such response, and opened new doors to the development of an improved B-cell-depletion strategy in terms of efficiency and duration.

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References
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