CD38 induces apoptosis of a murine pro-B leukemic cell line by a tyrosine kinase-dependent but ADP-ribosyl cyclase- and NAD glycohydrolase-independent mechanism

Frances E. Lund1, Hélène Muller-Steffner2, Héctor Romero-Ramirez3, Miguel E. Moreno-García3, Santiago Partida-Sánchez1, Melissa Makris1, Norman J. Oppenheimer4, Leopoldo Santos-Argumedo3 and Francis Schuber2

1Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983, USA
2Institut Gilbert Laustriat, Département de Chimie Bioorganique UMR 7175 CNRS/ULP, Strasbourg-Illkirch, France
3Departamento de Biomedicina Molecular, CINVESTAV-IPN, Mexico D.F., CP 07360 Mexico
4Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA 94143, USA

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Abstract

Cross-linking of CD38 on hematopoietic cells induces activation, proliferation and differentiation of mature T and B cells and mediates apoptosis of myeloid and lymphoid progenitor cells. In addition to acting as a signaling receptor, CD38 is also an enzyme capable of producing several calcium-mobilizing metabolites, including cyclic adenosine diphosphate ribose (cADPR). It has been previously postulated that the calcium-mobilizing metabolites produced by CD38 may regulate its receptor-based activities. To test this hypothesis, we examined whether the enzyme activity of CD38 controls the apoptosis of an anti-CD38-stimulated leukemic B cell. We show that anti-CD38-induced apoptosis of Ba/F3 cells, a murine pro-B cell line, is not affected by blocking the calcium-mobilizing activity of cADPR or by inhibiting intracellular or extracellular calcium mobilization. In addition, we demonstrate that blocking CD38 enzyme activity with 2'-deoxy-2'-fluoro-nicotinamide arabinoside adenine dinucleotide has no effect on apoptosis and that Ba/F3 cells expressing catalytically inactive mutant forms of CD38 still undergo apoptosis upon CD38 cross-linking. Instead, we find that anti-CD38-induced apoptosis is dependent on tyrosine kinase and caspase activation, and that this process appears to be potentiated by the presence of membrane microdomains. Thus, the receptor-mediated functions of CD38 can be separated from its enzyme activity in a murine leukemic cell line, suggesting that CD38 plays multiple, but independent, biologic roles.

Introduction

CD38 is a highly conserved type II transmembrane glycoprotein that is expressed on most bone marrow-derived cells (1, 2). Antibodies to human CD38 have been used clinically for many years to classify different B cell leukemias and lymphomas (3–5), and the cell-surface expression of CD38 on B-CLL cells is considered an excellent prognostic indicator for aggressive disease (6–8). However, the functional significance of CD38 expression on normal or malignant B lymphocytes is still not well understood. A number of groups have shown that cross-linking of CD38 using antibodies can induce a diverse array of biologic responses in mouse and human lymphocytes. For example, ligation of CD38 has been shown to induce aggregation of lipid raft microdomains, activation of kinases and calcium mobilization (reviewed in 2, 9). These signals can subsequently mediate the activation, proliferation and differentiation of mature B and T cells (10–17) as well as the growth and survival of leukemic cells (18–21). In addition, anti-CD38 stimulation has been shown to induce the apoptosis and death of both normal and leukemic progenitor B cells (22–28). Thus, cross-linking of CD38 induces pleiotropic effects on lymphocytes, even within the same lineage.

Based on the in vitro studies described above, CD38 clearly functions as a signaling receptor in lymphocytes. However, it is less clear how CD38 mediates signaling in B lymphocytes...
since the cytoplasmic tail of CD38 is very short (19–21 amino acids) and does not have any known docking sites for signaling proteins. However, CD38 still has the potential to mediate signal transduction because CD38 is an ecto-enzyme that can produce three known calcium-mobilizing metabolites (29). For example, the extracellular domain of CD38 binds to its substrate nicotinamide adenine dinucleotide (NAD⁺) and catalyzes the formation of cyclic adenosine diphosphate ribose (cADPR) and adenosine diphosphate ribose (ADPR) (30). cADPR mediates intracellular calcium release from ryanodine receptor-gated stores and induces the influx of extracellular calcium (29). ADPR, in contrast, has been shown to induce calcium influx through TRPM2 channels (31–34). In addition to producing cADPR and ADPR, CD38 can also catalyze a base-exchange reaction between nicotinamide adenine dinucleotide phosphate and nicotinic acid to produce nicotinic acid adenine dinucleotide phosphate (NAAD⁺) (35) which can also induce ryanodine receptor-mediated intracellular calcium release (36, 37).

The potential for the metabolites produced by CD38 to regulate calcium mobilization in cells that have been activated upon ligation of a variety of different types of receptors has been extensively investigated. For example, experiments with pancreatic acinar and beta cells isolated from CD38-deficient mice have demonstrated that CD38 and cADPR are required for calcium signaling in response to glucose and acetylcholine (38, 39). Likewise, activation of smooth muscle cells with agonists such as acetylcholine, endothelin-1 and oxytocin is also regulated by CD38-derived cADPR (40, 41). Similarly, we have shown that cADPR production by CD38 modulates calcium signaling through the G-protein-coupled chemokine receptors expressed by neutrophils, monocytes and dendritic cells (DCs) (42–44). While these data clearly show that CD38, through its production of cADPR, can regulate calcium signaling in cells activated by ligands for a number of G-protein-coupled receptors, it remains controversial as to whether the biologic activities observed after CD38 cross-linking are also dependent on the catalytic activity of CD38. On the one hand, a number of groups have shown that cross-linking of CD38 on lymphoid and myeloid cells induces calcium mobilization (10, 18, 28, 45, 46), consistent with the hypothesis that the enzymatic products generated by CD38 may mediate calcium signaling upon ligation of CD38. On the other hand, we previously demonstrated that anti-CD38-induced cytokine production occurred normally in a mature B cell line expressing a mutant form of CD38 with greatly reduced catalytic activity (47). Likewise, the Campana group showed that addition of NAD⁺, cADPR or ADPR to cultures of anti-CD38-stimulated progenitor B cells had no effect on the anti-CD38-induced apoptosis of these cells (22), suggesting that anti-CD38-induced signaling in B cells must occur independent of CD38 enzyme activity. However, these results were considered preliminary since the experiments were performed before all the metabolites produced by CD38 had been fully characterized and prior to the identification of the catalytic residue in the CD38 active site.

Since it has remained an open question whether CD38 enzyme activity is required for anti-CD38-induced signaling in B lymphocytes, we have now utilized an in vitro cell culture model to examine the signaling requirements for anti-CD38-induced apoptosis of leukemic pro-B cells. Using a cADPR antagonist, a specific inhibitor of CD38 enzyme activity, and cells expressing catalytically inactive forms of CD38, we show that anti-CD38-induced apoptosis of a progenitor B cell line occurs independent of CD38 enzyme activity. Instead, we find that anti-CD38-induced apoptosis of the progenitor B cells occurs via a tyrosine kinase- and caspase-dependent pathway and appears to be facilitated by the presence of microdomains associated with the plasma membrane. Together, these data indicate that the enzyme activity of CD38 is not required for anti-CD38-induced apoptosis of a B cell line and suggest that CD38 must have evolved two separate functions, first as an enzyme, which through its action on NAD generates metabolites that regulate calcium signaling, and second as a receptor, which when engaged by its ligand can induce signaling independent of its enzymatic activity.

Methods

Cell lines

The IL-3-dependent murine pro-B cell line Ba/F3 was obtained as a generous gift from Dario Campana (St Jude Children’s Hospital, Memphis, TN, USA). Ba/F3 cells were cultured in RPMI 1640 supplemented with either 10% WEHI-3 supernatant (containing IL-3) or recombinant IL-3 (2 ng ml⁻¹, R&D, Minneapolis, MN, USA), 10% FBS (GIBCO BRL, Rockville, MD, USA), non-essential amino acids, sodium pyruvate, L-glutamine, HEPES, mercaptoethanol and antibiotics. COS-7 cells were obtained from the American Type Tissue Collection and cultured in DMEM supplemented with 10% FBS, sodium bicarbonate, non-essential amino acids and HEPES.

Antibodies and inhibitors

Purified and fluorochrome-conjugated rat IgG2a anti-mouse monoclonal CD38 antibody (clone NIM-R5) was produced by the Trudeau monoclonal antibody facility. This mAb recognizes the extracellular domain of the native full-length murine CD38 (10). 8-bromo-cyclic adenosine diphosphate ribose (8-Br-cADPR), EGTA, methyl-β-cyclodextrin (mβCD) and aurintricarboxylic acid (ATA) were purchased from Sigma (St Louis, MO, USA). BAPTA-AM was purchased from Molecular Probes (Eugene, OR, USA), herbimycin A was from CalBiochem (La Jolla, CA, USA) and 2′-deoxy-2′-fluoro-nicotinamide arabinoside adenine dinucleotide (araF-NAD⁺) was synthesized as previously described (48).

DNA constructs

The expression vectors (CD38-pME18S/neo) containing the full-length membrane-bound murine CD38 (CD38-WT) and membrane-bound CD38-E229Q were generated by PCR using CD38-pME18S/neo as a template and the primers listed below (restriction sites are underlined, and the altered nucleotides that correspond to the replacement amino acid codons are indicated in lower case italics)—primer 1: 5′-TTT GGA AGT CTG cAg GTC TTT AGT-3′, primer 2: 5′-CCC TCT AGA cCA GAT CCT TCA CGT ATT AAG TCT ACA CG-3′, primer 3: 5′-ACT AAA GAC CTG cAg ACT TCC AAA GGT-3′.
and primer 4: 5′-GGG GAA TTC ATG GCT AAC TAT GAA TTT AGC CAG-3′.

The two PCR products were purified, digested with PstI, XbaI and EcoRI and then ligated into pME18S/neo to generate full-length CD38-E229Q. The entire CD38-coding region was then sequenced in both directions to ascertain that the appropriate mutation was introduced and that no polymerase or cloning errors were present in the rest of the cDNA.

To produce soluble CD38-WT, CD38-E150L and CD38-E229Q, the extracellular domain of CD38 was PCR amplified from expression plasmids containing full-length coding sequence of CD38-WT, CD38-E150L or CD38-E229Q using the primers listed below (restriction sites are underlined and CD38 sequence is in bold)—prime 1: 5′-GGACTAGTCTAAGGCC-GGCCTCAGTCTGGTGCTGAAGG-3′ and primer 2: 5′-GGATTAA-TTCGAGCTCGGTAC-3′.

The PCR products were purified and cloned into the L/FLAG pME18Sneo vector that contains the CD8α signal sequence and a FLAG tag (30).

Transfections
Ba/F3 cells were stably transfected with 25 μg linearized plasmid DNA by electroporation and selected in 500 μg ml⁻¹ Geneticin (GIBCO/BRL, Carlsbad, CA, USA) as previously described (50). After 10 days, the surviving CD38⁺ cells were cloned in 96-well plates using a FACS Vantage (BD Biosciences, San Jose, CA, USA). At least 20 independent clones from each transfection were tested for CD38 expression levels and at least five individual clones were picked to expand and analyze experimentally.

COS-7 cells were transiently transfected with 30 μg plasmid DNA per 10-cm dish using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The transfected cells were cultured for 72 h in serum-free DMEM.

Isolation, purification and quantitation of soluble recombinant WT and mutant CD38 protein
Culture medium from transiently transfected COS-7 cells was adjusted to 150 mM NaCl, centrifuged and filtered. The medium was passed over an anti-FLAG M2 affinity gel column (Sigma) overnight at a rate of 4 ml min⁻¹. The column was washed with Tris-buffered saline (TBS), and the FLAG-tagged proteins were eluted using purified FLAG peptide (Sigma) at 150 μg ml⁻¹ in TBS. Eluted proteins were collected and concentrated on a Millipore 10 000 MWCO spin filter (Fisher, Houston, TX, USA) to a final volume of 100 μl.

To quantitate the FLAG-tagged recombinant CD38 protein, ELISAs and Bradford assays were performed. Bradford assays were performed using a microassay protocol with BSA as the standard (Biorad, Hercules, CA, USA). ELISA plates were coated with 2 μg ml⁻¹ anti-CD38 mAb (NIM-R5), the FLAG-tagged proteins were applied to the plate, biotinylated anti-FLAG M2 antibody (2 μg ml⁻¹, Sigma) was added and streptavidin–HRP (Southern Biotechnology, Birmingham, AL, USA) was used as the developing reagent. FLAG-tagged recombinant WT soluble CD38 was used as the standard.

Apoptosis assays
Annexin-V stain. Parental CD38neo or CD38 transfected Ba/F3 cells were cultured (2 × 10⁵ cell ml⁻¹) in Ba/F3 media in the presence of anti-CD38 or a rat IgG2a isotype control antibody. Stimulation of CD38-expressing Ba/F3 cells with 1–10 μg ml⁻¹ anti-CD38 induced an equivalent maximal apoptotic response. Cells were collected between 1 and 18 h post-stimulation and immediately stained with Annexin-V FITC and propidium iodide (PI) according to the manufacturer’s directions (Clontech, Palo Alto, CA, USA). The cells were analyzed using a FACS Calibur flow cytometer and Cell Quest (BD Bioscience) or FlowJO (Treestar, Boston, MA, USA) software. The percentage of Annexin-V⁺ cells was maximal within 1–2 h of anti-CD38 stimulation. At later time points (>4 h), the percentage of Annexin-V⁺ cells was similar to the earlier 4-h time point but most of the Annexin-V⁺ cells also stained with PI, indicating that the cells were dying or dead. In most cases, the data are shown as the percentage of total Annexin-V⁺ cells which include both the Annexin-V⁺/PI⁻ (apoptotic) and the Annexin-V⁺/PI⁺ (dying or dead) cells.

TUNEL assay. The TUNEL assay was performed using the In Situ Cell Death Detection Kit tetramethylrhodamine (TMR-red) (Roche, Penzberg Germany) according to the manufacturer’s instructions. Briefly, after incubation with anti-CD38 or control antibody, the cells (2 × 10⁶ per sample) were fixed in 100 μl 4% PFA (pH 7.4) for 1 h at room temperature. The cells were washed, re-suspended in 100 μl 0.1% Triton X-100 and 0.1% sodium citrate and incubated for 2 min on ice. The cells were washed and re-suspended in freshly prepared terminal deoxynucleotidyl transferase (TdT) and TMR-labeled nucleotides. The negative controls were treated with the labeled nucleotides without TdT. The plates were incubated in the dark for 1 h at 37°C, washed and analyzed using a FACS Calibur.

Caspase activity assay. Activated caspases present in the stimulated Ba/F3 cells were detected with the ApoFluor Green caspase activity assay kit (ICN, Irvine, CA, USA) using either carboxyfluorescein (FAM)-labeled valylalanylaspartic acid fluoromethyl ketone (VAD-FMK) to detect all activated caspases or FAM-DEVD-FMK to detect activated caspase-3 and caspase-3-like caspases. Briefly, cells were incubated for 4 h at 3 × 10⁶ ml⁻¹ in the presence of 5 μg ml⁻¹ anti-CD38 or control antibody. As a positive control for apoptosis induction, additional cultures of Ba/F3 transfectedants were deprived of IL-3 for 19 h. Cells were harvested and labeled with the ApoFluor Green reagents according to the manufacturer’s recommendations. Samples were analyzed in a 96-well fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 488 nm and emission wavelength of 515 nm. Data are reported in relative fluorescence units (RFUs).

Apoptosis inhibition assays
Calcium chelators. Parental CD38neo or CD38 transfected Ba/F3 cells and CD38-WT transfected cells (2 × 10⁵ ml⁻¹) were incubated in HBSS (no Ca²⁺ or Mg²⁺) in the presence of 10% WEHI-3 supernatant (as a source of IL-3), 3% BSA and EGTA (5 mM) or BAPTA-AM (20 μM). The medium containing EGTA was buffered to pH 7.4 with 100 mM HEPES. The cells were stimulated in the presence or absence of anti-CD38 (NIM-R5, 2.5 μg ml⁻¹) for 4 h.
Tyrosine kinase and endonuclease inhibitors. A total of 1 x 10^6 cells were pre-incubated with different concentrations of ATA, herbimycin A or the corresponding vehicles [PBS for ATA and dimethyl sulfoxide (DMSO) for herbimycin]. DMSO at 0.5 μl/ml was the highest concentration used in any experiment. After 1 h at 37°C, the cells were treated with anti-CD38 or control antibodies as described above. Cells incubated with the drugs or the vehicle, in the absence of anti-CD38, were >90% viable at the end of the experiments.

Measurement of cyclase and glycohydrolase activity of soluble CD38 proteins

The NAD+ glycohydrolase and ADP-ribosyl cyclase activity of recombinant soluble CD38 proteins were measured under saturating conditions using [adenosine-U-14C]NAD+ (5 x 10^5 d.p.m.) as previously described (47). Purified and quantitated FLAG-tagged soluble CD38 protein was suspended in 200 μl potassium phosphate buffer (50 mM, pH 6.8) and incubated at 37°C with the substrate. At selected times, aliquots (50–100 μl) were removed and enzyme activity was stopped by adding ice-cold perchloric acid (2% final concentration). Precipitated proteins were removed by centrifugation and the supernatant was neutralized with 3.5 M K2CO3. Samples were then analyzed by HPLC on a 300 x 3.9-mm μBondapack C18 column (Waters Assoc., Milford, MA, USA). The compounds were eluted isocratically with a 10 mM ammonium phosphate buffer, pH 5.5, containing 0.8–1.2% (v/v) acetonitrile and detected by radiodetection (Flo-one, Packard Radiometric Instruments, Meriden, CT, USA). Standards were injected to determine retention times and identify the reaction products. Using this assay method, transformation of NAD+ as low as 5 pmol min⁻¹ can be detected.

Results

Cross-linking CD38 induces cell death in a leukemic B cell line

Previous publications from the laboratory of D. Campana showed that transfection of the mouse progenitor B cell line Ba/F3 with human CD38 rendered the cells susceptible to cell death after stimulation with anti-human CD38 antibody (26). To test whether the expression of murine CD38 in Ba/F3 cells would confer susceptibility to cell death after cross-linking CD38, parental CD38-negative Ba/F3 cells (parental CD38neg) were first transfected with full-length murine CD38 (CD38-WT). Stable transfectant clones were isolated, and cells expressing high levels of murine CD38 were FACS sorted and subcloned. CD38 expression levels on representative Ba/F3 clones expressing CD38-WT and parental CD38neg Ba/F3 cells are shown in Fig. 1(A). The CD38-expressing and non-expressing clones were then stimulated with the anti-mouse CD38 mAb NIM-R5 or an isotype control antibody for 6 h in media containing IL-3, an essential growth factor for Ba/F3 cells. The clones were then stimulated with anti-CD38 or the isotype control antibody. Likewise, stimulation of the WT-CD38-expressing Ba/F3 cells with an isotype control antibody (Fig. 1B and C) or antibodies to an irrelevant protein (data not shown) did not induce the cells to become Annexin-V⁻. In contrast, ~60% of the CD38-WT transfected Ba/F3 cells were Annexin-V⁻PI⁻ (potentially apoptotic) or Annexin-V⁻PI⁺ (dying or dead) within 6 h of anti-CD38 stimulation (Fig. 1B and C).

We observed similar results when the cells were stimulated for 4–24 h using anti-CD38 (NIM-R5) at a concentration of 1–10 μg ml⁻¹, when we used other monoclonal or polyclonal anti-CD38 reagents and when we used an Fab'2 anti-CD38 antibody (data not shown). Furthermore, essentially no live cells were
recovered from the anti-CD38-stimulated, CD38-WT-expressing Ba/F3 cultures after 48 h of stimulation, while the anti-CD38-stimulated parental Ba/F3 cells expanded normally (Fig. 1D). Importantly, parental CD38\textsuperscript{neg} and CD8-WT transfected Ba/F3 cells incubated with isotype control antibody or in the absence of any antibody proliferated equivalently over the 2-day culture period (Fig. 1D). The same results were observed with several independently isolated CD38-WT-expressing Ba/F3 cells (data not shown). Together, these data indicate that murine pro-B leukemic cells will die in culture after CD38 cross-linking.

**CD38 ligation induces DNA damage and caspase activation**

Cross-linking CD38 on Ba/F3 cells induced re-organization of the plasma membrane and re-distribution of phosphatidylserine to the outer layer of the membrane, one of the early signs of a cell undergoing apoptosis (51). To determine whether anti-CD38 treatment induced DNA breaks, another hallmark of an apoptotic cell (51), we incubated parental CD38\textsuperscript{neg} and CD38-WT transfected cells with anti-CD38 or an isotype control antibody for 1 h and then measured TUNEL activity using TMR-labeled nucleotides and TdT. As shown in Fig. 2(A), only CD38-WT Ba/F3 cells stimulated with anti-CD38 incorporated TMR-labeled nucleotides in the presence of TdT enzyme, indicating that DNA breaks occurred in these cells rapidly after stimulation and strongly suggesting that anti-CD38 stimulation induced apoptosis of the progenitor B cells.

Next, to test whether the DNA damage induced in the anti-CD38-stimulated Ba/F3 cells was responsible for the death of the cells, we pre-incubated the Ba/F3 cells in the presence of increasing concentrations of the endonuclease inhibitor ATA or a solvent control (PBS) and then stimulated the cells with anti-CD38 or an isotype control antibody. We then determined the percentage of cells that were apoptotic, dying or dead. As shown in Fig. 2(B), we observed a significant reduction in the percentage of dying cells when the endonuclease inhibitor was included in the cultures, indicating that anti-CD38-stimulated Ba/F3 cells were dying by a DNA damage-dependent mechanism.

Apoptotic cell death is usually dependent on the activation of caspases (51). To test whether caspase activation occurred after anti-CD38 stimulation, we stimulated CD38-WT transfected and parental CD38\textsuperscript{neg} Ba/F3 cells with anti-CD38 or an...
isotype control antibody and then measured caspase activation using a fluorescently labeled caspase inhibitor (VAD-FMK) that binds irreversibly to all activated caspases. As a positive control for caspase activation, we also cultured cells in the absence of their obligate growth factor, IL-3. As expected, caspase activation occurred in both the CD38 transfected and parental Ba/F3 cells that were deprived of IL-3 (data not shown). In contrast, caspases were not activated in the parental CD38neg Ba/F3 cells stimulated with anti-CD38 (Fig. 2C). However, we observed strong caspase activation in CD38 transfected Ba/F3 cells that were stimulated with anti-CD38 but not with control antibody (Fig. 2C). To identify which caspase pathway was activated after CD38 ligation, we then measured the activation of individual caspases using fluorescent inhibitors for specific caspases. Interestingly, using fluorescently labeled DEVD-FMK, we found that caspase-3 and caspase-3-like caspases were modestly activated in anti-CD38-stimulated, CD38-WT-expressing Ba/F3 cells when compared with the CD38-WT-expressing cells that were treated with an isotype control antibody (Fig. 2C). In contrast, despite a higher background caspase-3 activity in the parental CD38neg Ba/F3 cells, no significant further activation was seen after treatment of these cells with anti-CD38. Thus, the data suggest that caspase-3 is one of the possibly multiple caspases that are activated after CD38 cross-linking. Taken altogether, these data show that cross-linking CD38 in a leukemic cell line induces activation of capases, DNA damage and apoptotic cell death.

An antagonist of cADPR does not block anti-CD38-induced cell death

Previous studies have shown that anti-CD38-induced cell death in leukemic cells is not dependent on sequences in the cytoplasmic tail of CD38 (26), suggesting that the extracellular domain of CD38 may facilitate activation of the intracellular tyrosine kinases. Since the ectodomain of CD38 can catalyze the formation of calcium-mobilizing metabolites such as cADPR (52), we hypothesized that these metabolites might induce apoptosis. To test this possibility, we stimulated parental

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**Fig. 2.** Cross-linking of CD38 on Ba/F3 cells induces caspase activation and apoptotic cell death. (A) TUNEL staining was performed on parental CD38neg and CD38-WT Ba/F3 cells that were incubated for 1 h with anti-CD38 or isotype control antibody (1 μg ml⁻¹). Cells were incubated with TMR-red-labeled nucleotides in the presence (filled histogram) or absence of TdT (dotted line histogram) as described in Methods. Unstained control cells are shown in the solid line histogram. The percentage of cells incorporating TMR-red is indicated. The results are representative of two independent experiments. (B) CD38-WT Ba/F3 cells were pre-incubated for 1 h with 0.5 mM ATA or vehicle control (PBS), stimulated for a further 1 h with anti-CD38 or control antibody (1 μg ml⁻¹) and then stained with Annexin-V and PI. Data are representative of three independent experiments. (C) Parental CD38neg and CD38-WT Ba/F3 cells were incubated in triplicate cultures for 4 h in the presence of anti-CD38 or control antibody (5 μg ml⁻¹). Caspase activation in the different cell samples was measured by incubating the cells with fluorescently labeled VAD-FMK (a pan-caspase inhibitor, top) or DEVD-FMK (a caspase-3 inhibitor, bottom). The data are shown in RFUs and are reported as the mean ± standard deviation of triplicate cultures. Data are representative of two to three independent experiments. P values were determined by Student's t-test.
CD38<sup>reg</sup> Ba/F3 cells and CD38-WT transfected Ba/F3 cells with anti-CD38 in the presence or absence of a competitive antagonist for cADPR, 8-Br-cADPR. The 8-Br-cADPR is a membrane-permeant analog of cADPR that specifically blocks cADPR-dependent calcium release in a number of cell types (53), including neutrophils and DCs (44). As shown in Fig. 3, parental CD38<sup>reg</sup> Ba/F3 cells did not become Annexin-V<sup>+</sup> after stimulation with anti-CD38 or anti-CD38 + 8-Br-cADPR. Furthermore, treatment of these cells with 8-Br-cADPR did not induce cell death (Fig. 3), indicating that 8-Br-cADPR does not block IL-3R-dependent signaling. As expected, anti-CD38 stimulation of the CD38-WT transfected Ba/F3 cells induced cell death, with >40% of the cells staining with Annexin-V and PI (Fig. 3). However, the presence of 8-Br-cADPR in the anti-CD38-stimulated cultures had no effect on anti-CD38-induced cell death (Fig. 3). Although 8-Br-cADPR treatment had no effect on anti-CD38-induced apoptosis, the 8-Br-cADPR used in these experiments was biologically active as treatment of primary neutrophils with the same dose of compound (~20-fold above the 50% inhibitory concentration of the compound in leukocytes (43)) effectively blocked chemokine receptor signaling (data not shown). Therefore, these data suggest that cross-linking CD38 does not induce apoptosis via a cADPR-dependent pathway.

**Inhibition of CD38 enzyme activity does not block anti-CD38-induced apoptosis**

cADPR is not the only calcium-mobilizing metabolite produced by CD38. Indeed, the predominant product of the CD38 enzyme reaction, ADPR, induces calcium influx through TRPM2 channels in monocytes (31–34). In addition, a product of the CD38-dependent base-exchange reaction, NAADP<sup>+</sup>, has been shown to mediate intracellular calcium release through IP<sub>3</sub>R-independent stores in a number of cell types (29), including T lymphocytes (54). In order to test whether any of the metabolites generated by CD38 induce cell death in anti-CD38-stimulated progenitor cells, we blocked the enzyme activity of CD38 by incubating parental CD38<sup>reg</sup> Ba/F3 cells and CD38-WT Ba/F3 transfectants with a CD38 substrate analog, araF-NAD<sup>+</sup>. araF-NAD<sup>+</sup>, the most powerful known competitive inhibitor of human CD38 (K<sub>i</sub> = 1.7 nM), prevents CD38-dependent product formation by blocking the binding of NAD<sup>+</sup> to the CD38 active site (55–57). After incubating CD38<sup>reg</sup> Ba/F3 cells or CD38-WT transfected Ba/F3 cells in the presence or absence of araF-NAD<sup>+</sup> for 1 h, an aliquot of the cells was removed and CD38-dependent enzyme activity was measured. The remaining cells were cultured with anti-CD38 or isotype control antibodies for an additional 4 h. An aliquot of the cells was removed at the end of the culture period and CD38-dependent enzyme activity was again measured. As expected, CD38-WT-expressing Ba/F3 cells, but not the parental CD38<sup>reg</sup> control cells, hydrolyzed the CD38 substrate ε-NAD<sup>+</sup> to the highly fluorescent product ε-ADPR (Fig. 4A). However, after 1 h of incubation with the CD38 inhibitor, araF-NAD<sup>+</sup>, CD38-WT-expressing Ba/F3 cells were unable to hydrolyze ε-NAD<sup>+</sup> and did not produce detectable quantities of ε-ADPR. Likewise, after the CD38-WT transfected cells were incubated for an additional 4 h in the presence of control or anti-CD38 antibodies and araF-NAD<sup>+</sup>, the CD38-WT transfected cells remained enzymatically inactive (Fig. 4B). Thus, under our experimental conditions, treatment of the cells with araF-NAD<sup>+</sup> totally inhibited CD38 enzyme activity throughout the experimental time course.

To determine whether the araF-NAD<sup>+</sup> would block anti-CD38-induced cell death, the apoptotic profile of the cells stimulated with anti-CD38 in the presence or absence of araF-NAD<sup>+</sup> was determined. Approximately 10% of the parental CD38<sup>reg</sup> Ba/F3 cells were Annexin-V<sup>+</sup>. This percentage of dying cells remained constant regardless of whether the cells were left in media alone, treated with araF-NAD<sup>+</sup>, stimulated with anti-CD38 alone or treated with araF-NAD<sup>+</sup> and then stimulated with anti-CD38 (Fig. 4C). In contrast, 35–40% of the Ba/F3 cells transfected with CD38-WT became Annexin-V<sup>+</sup> after anti-CD38 stimulation. Furthermore, continuous incubation of the cells with araF-NAD<sup>+</sup> did not block anti-CD38-induced cell death (Fig. 4C). Thus, although araF-NAD<sup>+</sup> treatment completely inhibited CD38 enzyme activity, it had no effect on anti-CD38-induced apoptosis of the Ba/F3 cells.

**Ba/F3 cells expressing a catalytically inactive form of CD38 still undergo anti-CD38-induced apoptosis**

The results presented above suggested that CD38 catalytic activity may not be required for anti-CD38-mediated signaling in Ba/F3 cells. However, since CD38 is a constitutively active enzyme, it is possible that the enzymatic products could be produced and subsequently stored in the cells. If this were true, then acute inhibition of CD38 enzyme activity with araF-NAD<sup>+</sup> may not be sufficient to block signaling induced
by ‘pre-formed’ pools of CD38-generated metabolites. To ensure that no CD38-catalyzed products could be formed and then stored in the cells, we expressed enzymatically inactive mutants of CD38 in Ba/F3 cells. We previously showed that the glutamate at position 150 in murine CD38 is an important active site residue as mutations at this site reduced CD38-dependent enzyme activity by >95% (47). In addition, Lee and co-workers have shown for human CD38 that the glutamate at position 226 (229 in murine CD38) is absolutely essential for enzyme activity because mutations at this position in human CD38 completely abolished enzyme activity (58). To test whether mutations of either of these two residues would render murine CD38 enzymatically inactive, we replaced the glutamate at position 150 with a leucine (CD38-E150L) and the glutamate at position 229 with a glutamine (CD38-E229Q). We expressed and purified soluble recombinant forms of these proteins and then determined the specific activity of the recombinant proteins in CD38-dependent enzyme assays using radiolabeled NAD+ as the substrate. As expected based on our previous results (47), the soluble recombinant CD38-E150L protein had greatly decreased, although still detectable, enzyme activity compared with soluble recombinant CD38-WT protein (Table 1). In contrast, no catabolites were detected when NAD+ was incubated with CD38-E229Q protein, even though a very sensitive radioactive-based HPLC assay was utilized (Table 1). Thus, similar to the previously published observations with human CD38-E226Q (58), we found that the murine CD38 amino acid E229 is a critical catalytic residue.

In order to test whether cells expressing the catalytically defective form of CD38 would undergo apoptosis after anti-CD38 stimulation, we stably expressed the full-length membrane-bound forms of CD38-E229Q and CD38-E150L in Ba/F3 cells. The membrane expression levels of the mutant CD38 proteins were approximately equivalent to CD38-WT transfected cells (data not shown), indicating that the mutant forms were efficiently translated and transported to the plasma membrane. Membrane homogenates were prepared from the transfected cells, and as expected, the CD38-dependent enzyme activities of cells expressing the two membrane-bound mutant forms of CD38 were at or below the threshold of detection (data not shown).

To determine whether cross-linking of the catalytically defective CD38 molecules on Ba/F3 cells would induce apoptosis, parental CD38<sup>neg</sup> Ba/F3 cells and CD38-WT Ba/F3 cells were stimulated for 8 h in the presence or absence of anti-CD38 antibody. The cells were then stained with Annexin-V FITC and...
PI and the percentage of Annexin-V+ cells was determined. A representative experiment is shown in Fig. 5. Similar to our earlier experiments, cells stimulated with an isotype control antibody did not become apoptotic (Fig. 5A) and were indistinguishable from cells cultured in IL-3-containing media alone (data not shown). As expected, anti-CD38-stimulated parental CD38neg Ba/F3 cells did not undergo apoptosis, while ~50% of the anti-CD38-stimulated Ba/F3 cells expressing CD38-WT were apoptotic or dead within 8 h (Fig. 5A). Interestingly, 50% or more of the cells expressing either the CD38-E150L or the CD38-E229Q mutant protein also underwent apoptosis upon stimulation with anti-CD38 (Fig. 5A). On average, we observed a 7- to 12-fold increase in the percentage of apoptotic cells after anti-CD38 stimulation in cells expressing CD38-WT as well as CD38-E150L and CD38-E229Q and we saw no significant differences in the anti-CD38-induced apoptotic response between any of the different transfectants (Fig. 5B). Taken altogether, these data demonstrate that anti-CD38-induced apoptosis of progenitor Ba/F3 cells occurs independent of CD38 enzyme activity.

Table 1. Enzyme activity of soluble recombinant CD38

<table>
<thead>
<tr>
<th>Soluble</th>
<th>Enzyme activityb</th>
<th>% of CD38-WT enzyme activityc</th>
</tr>
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<tbody>
<tr>
<td>CD38-WT</td>
<td>203.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CD38-E150L</td>
<td>9.7</td>
<td>4.8</td>
</tr>
<tr>
<td>CD38-E229Q</td>
<td>&lt;0.02</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Soluble recombinant CD38-WT, CD38-E150L and CD38-E229Q were expressed and purified as described in Methods. bThe enzyme activity of recombinant soluble CD38 proteins was determined under saturating conditions (Vmax) using [adenosine-U-14C]NAD+ as the substrate. The activity of the E229Q mutant was less than our detection limit of 5 pmol min−1. cThe activity of CD38-WT was set at 100% and the relative enzyme activity of the mutant proteins compared with CD38-WT is shown.

Anti-CD38-induced apoptosis can occur in the absence of intracellular or extracellular free calcium

Our results indicated that anti-CD38-mediated signaling in progenitor B cells occurs independent of CD38 enzyme activity, suggesting that calcium signaling may not be required for anti-CD38-induced apoptosis. To test this hypothesis, we stimulated parental CD38neg Ba/F3 cells and CD38-WT-transfected Ba/F3 cells with anti-CD38 in the presence of the calcium chelators, BAPTA-AM or EGTA. EGTA is relatively membrane impermeant and therefore chelates free extracellular calcium, while BAPTA-AM is membrane permeant and will chelate intracellular calcium. The parental CD38neg Ba/F3 cells did not develop an apoptotic phenotype when incubated in BAPTA-AM (Fig. 6A) or EGTA (Fig. 6B) in the presence or absence of anti-CD38. In contrast, CD38-WT-expressing Ba/F3 cells rapidly underwent apoptosis when stimulated with anti-CD38 (Fig. 6A and B). However, neither BAPTA-AM (Fig. 6A) nor EGTA (Fig. 6B) had any effect on the apoptotic response of anti-CD38-stimulated CD38-WT Ba/F3 cells. Although treatment of the CD38-WT-expressing cells with EGTA or BAPTA-AM had no effect on the apoptotic response of the anti-CD38-stimulated CD38-WT-expressing cells, treatment of primary neutrophils with the same dose of these chelating reagents very efficiently blocked chemotactic responses (data not shown). Therefore, these data strongly suggest that anti-CD38-induced apoptosis can occur in the absence of calcium mobilization from intracellular or extracellular sources.

Anti-CD38-induced apoptosis is regulated by tyrosine kinase activation and plasma membrane microdomains

Our data clearly demonstrated that CD38 cross-linking induces apoptosis of leukemic B cells by a mechanism that is independent of CD38 enzyme activity and calcium signals. These findings were somewhat surprising as the intracellular cytoplasmic tail of CD38 can be truncated to a...
single amino acid or completely replaced without affecting the biologic responses induced after CD38 cross-linking (26, 47). Thus, it was not entirely clear how ligation of CD38 could induce intracellular signaling, caspase activation and apoptosis. As previously demonstrated, ligation of CD38 in primary human B cell progenitors and in Ba/F3 cells transfected with human CD38 results in the tyrosine phosphorylation and activation of a number of signaling molecules including Tec, cbl, PI3K, syk and PLCγ (23–26). Furthermore, incubation of the progenitor B cells with various kinase inhibitors, including the PI3K inhibitor Wortmannin, blocks the phosphorylation of target proteins after anti-CD38 stimulation (24). To determine whether tyrosine kinase activation is necessary for anti-CD38-induced apoptosis of Ba/F3 cells, we treated CD38-WT Ba/F3 cells with herbimycin A and then measured Annexin-V staining. Treatment of parental CD38neg with herbimycin A had no effect on the viability of the cells. However, herbimycin A very efficiently blocked anti-CD38-induced apoptosis (Fig. 7, bottom panel) and apoptotic cell death (Fig. 7, top panel) of the CD38-WT transfected Ba/F3 cells at a range of concentrations (data not shown), indicating that tyrosine kinase activation is required for anti-CD38-induced apoptosis.

Previous studies have shown that a significant proportion of the total CD38 molecules expressed on the plasma membrane of B and T cells are constitutively localized in specialized microdomains that are enriched in sphingolipids, cholesterol and glycosyl-phosphatidylinositol-anchored proteins (59, 60). Furthermore, upon cross-linking of CD38 in T and B lymphocytes, the lipid raft membrane domains coalesced (18, 59), and in T cells, this resulted in recruitment and activation of tyrosine kinases such as Akt (59). Therefore, we hypothesized that CD38 cross-linking might mediate tyrosine kinase activation and subsequent apoptosis by facilitating the aggregation of lipid rafts or by promoting the recruitment of signaling proteins.
molecules into the lipid rafts. To test this hypothesis, we pre-treated CD38 transfected cells for 2 h with mJCD, a compound that extracts plasma membrane cholesterol and disrupts lipid raft microdomains (61). We then added anti-CD38 to induce receptor cross-linking and apoptosis. After an additional 2 h, we measured apoptosis by staining with Annexin-V and PI. As shown in Table 2, treatment of the cells with 2 mM mJCD had no effect on anti-CD38-induced apoptosis in CD38-WT or parental CD38negBa/F3 cells. However, treatment of CD38-WT-expressing Ba/F3 cells with 5 mM mJCD did significantly reduce the percentage of apoptotic cells in the cultures. This was despite the fact that mJCD was toxic to the cells as it caused a significant increase in cell death in the parental CD38neg cells at the 5-mM concentration and almost completely killed both CD38-WT and the parental CD38neg cells at a 10-mM concentration (Table 2). Thus, disruption of the plasma membrane using a cholesterol-depleting agent appears to inhibit the ability of CD38 to induce apoptosis upon cross-linking. This result, in combination with our data demonstrating that the enzyme activity of CD38 is not required for the CD38-dependent induction of apoptosis in malignant cells, suggests that CD38 mediates its receptor-based functions in an indirect fashion, perhaps by causing aggregation and co-localization of tyrosine kinases within membrane microdomains.

Discussion

Over the last decade, a wide variety of functions have been assigned to CD38. In particular, cross-linking of CD38 using CD38-specific antibodies induces a number of biologic effects in lymphocytes including cellular activation as well as cell death (reviewed in 1, 2). In addition, CD38 appears to play an important role in regulating both the growth (18–20) and apoptosis (22, 28) of malignant B cells. In a series of elegant experiments from the Campana laboratory, they showed that anti-CD38 stimulation of human progenitor B cell lines induces phosphorylation and activation of a number of signaling molecules including lyn, syk, cbl, Tec and PI3K (23–25, 27). Furthermore, they found that inhibition of PI3K with Wortmannin significantly blocked the anti-CD38-induced apoptotic response in normal and leukemic progenitor B cells (24). In the experiments presented here, we show that cross-linking of CD38 on leukemic progenitor B cells leads to an apoptotic cell death that is induced upon caspase activation and is critically dependent on the presence of endonucleases and activated tyrosine kinases.

Despite the now established roles for tyrosine kinases and PI3K in regulating anti-CD38-induced apoptosis of leukemic B cells, the mechanism by which CD38 induces intracellular signaling is still unclear, particularly since the intracellular cytoplasmic domain of CD38 is dispensable for anti-CD38-mediated signaling (26, 47) and the transmembrane domain of CD38 can be completely replaced without altering CD38-mediated signaling (47). Therefore, given that the signals induced upon CD38 cross-linking appear to be controlled by the extracellular domain of CD38, it was certainly reasonable to propose that the receptor-mediated functions of CD38 might be controlled by the ecto-enzymatic activity of CD38. While earlier experiments from the Campana laboratory and our laboratory suggested that the catalytic activity of CD38 did not control B cell activation or apoptosis (22, 47), they were performed before more specific antagonists/inhibitors and CD38 mutants were available. However, the experiments in this study, using more sensitive and accurate methods to block CD38 enzyme activity, clearly demonstrate that the metabolites produced by the CD38 are not necessary for anti-CD38-induced signaling in at least one murine leukemic pro-B cell line.

First, we showed that the treatment of CD38-expressing Ba/F3 cells with an antagonist of cADPR-mediated calcium release (8-Br-cADPR) had absolutely no effect on anti-CD38-mediated apoptosis. To rule out the possibility that one of the other calcium-mobilizing metabolites produced by CD38, such as ADPR or NAADP*, was able to regulate calcium signaling after anti-CD38 stimulation, we treated CD38-expressing cells with a highly powerful and specific CD38 enzyme inhibitor (araF-NAD*) and then measured the apoptotic response after anti-CD38 stimulation. We showed that the araF-NAD*-treated cells had no detectable CD38-dependent enzyme activity, yet the araF-NAD*-treated cells became apoptotic after anti-CD38 stimulation with equal frequency as cells that were enzymatically competent. Finally, we expressed the catalytically inactive mutant CD38-E229Q in Ba/F3 cells and showed that anti-CD38-induced apoptosis at equivalent levels in the cells expressing CD38-E229Q compared with cells expressing CD38-WT. These data, taken together, indicate that the enzyme activity of CD38 does not control intracellular signaling after CD38 cross-linking in the murine Ba/F3 cell line.

Although apoptosis after CD38 cross-linking can occur in the absence of CD38 enzyme activity and the production of cADPR, ADPR or NAADP*, intracellular calcium mobilization induced upon production of other calcium-mobilizing second messengers, such as IP3, may still be needed to induce productive signaling after CD38 cross-linking. Indeed, many laboratories have shown calcium mobilization in response to anti-CD38 stimulation (10, 28, 46), and we have found that anti-CD38-mediated activation of mature B cells is critically dependent on extracellular calcium influx (45). However, at least for the progenitor Ba/F3 B cells, we found that the apoptotic response induced after CD38 cross-linking was not dependent on any source of calcium, whether from external or internal stores. Thus, while calcium mobilization may be an

Table 2. Anti-CD38-induced apoptosis in the presence of a lipid raft inhibitor

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>% of Annexin-V* CD38-WTa</th>
<th>% of Annexin-V* parental CD38neg</th>
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<tbody>
<tr>
<td>Nil</td>
<td>7.8 ± 0.8</td>
<td>10.5 ± 0.9</td>
</tr>
<tr>
<td>Anti-CD38 (5 μg ml−1)</td>
<td>63.5 ± 2.2</td>
<td>10.8 ± 0.7</td>
</tr>
<tr>
<td>Anti-CD38 + mJCD (2 mM)</td>
<td>59.8 ± 3.9</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td>Anti-CD38 + mJCD (5 mM)</td>
<td>44.8 ± 0.6c</td>
<td>19.8 ± 7.4c</td>
</tr>
<tr>
<td>Anti-CD38 + mJCD (10 mM)</td>
<td>88.4 ± 1.2d</td>
<td>85.0 ± 3.3d</td>
</tr>
</tbody>
</table>

*Cells were stimulated with anti-CD38 for 4 h in the presence of increasing concentrations of mJCD. The percentage of Annexin-V* cells was determined by FACS and the data are shown as mean ± standard error of the mean of three or more wells per group. bP = 0.0008 versus anti-CD38 alone using unpaired Student's t-test. cP = 0.018 versus anti-CD38 alone using unpaired Student's t-test. d5mJCD (10 mM) was toxic to both CD38-WT and parental CD38neg cells.
important component of the anti-CD38-induced signaling response in mature B cells, it is not required in this leukemic pro-B cell line.

If neither the enzyme activity nor the cytoplasmic tail of CD38 is necessary for anti-CD38-mediated signaling, how does CD38 function as a signaling receptor in lymphoid cells? It is clear from our work that activation of tyrosine kinases is critical for anti-CD38-mediated apoptosis of Ba/F3 cells and from the Campana laboratory very nicely showed that anti-CD38 stimulation of Ba/F3 cells leads to tyrosine phosphorylation of a number of key signaling molecules including Tec and cbl (26). Furthermore, our data suggest that either lipid rafts or other membrane structures affected by treatment with the cholesterol-depleting agent MβCD may facilitate anti-CD38-induced apoptosis in Ba/F3 cells. However, MβCD treatment causes cell membrane damage as seen by the fact that simply treating parental Ba/F3 cells with a low dose of MβCD caused an increase in the percentage of apoptotic cells (Table 2). While these data did not conclusively prove that CD38 signaling is dependent on the formation of lipid rafts, other groups showed that a proportion of CD38 is constitutively localized to the plasma membrane raft microdomains (59, 60). Upon anti-CD38 stimulation of T cells, the membrane rafts coalesce and enlarge, recruiting more TCR-associated signal transduction molecules to the lipid rafts, resulting in the activation of critical TCR-associated tyrosine kinases such as Zap-70 (17, 59, 62). Similar experiments performed with B-CLL cells also suggested that CD38 cross-linking leads to co-localization of CD38 and the BCR complex within the rafts (18). Based on these findings, we propose that CD38 may mediate its receptor-based signals indirectly by facilitating aggregation, co-localization and subsequent activation of critical tyrosine kinases in discrete membrane microdomains. This model is consistent with the published data showing that the cytoplasmic tail and transmembrane domain of CD38 are not required for signaling as CD38 would not need to be in direct physical contact with the cytoplasmic kinases or adaptor proteins to mediate signaling.

In summary, in contrast to our previous studies showing that CD38 regulates cell migration by catalyzing the production of calcium-mobilizing metabolites (42-44, 63), our new data indicate that receptor-mediated ligation of CD38 results in the apoptotic death of leukemic B cells via a tyrosine kinase- and caspase-dependent process that occurs independent of CD38 enzyme activity. Given that the calcium-mobilizing metabolites produced by CD38 play a critical role in signaling in a variety of cell types, including leukocytes (42, 44), our data suggest that CD38 has likely evolved into a ‘moonlighting’ enzyme that performs dual but independent roles in vivo, first as a generator of calcium-mobilizing metabolites and second as a plasma membrane signaling receptor. Finally, our data show that while antibodies to CD38 may be effective in inducing the apoptosis of CD38-expressing tumor cells, CD38 enzyme inhibitors are unlikely to be efficacious in this application.

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Abbreviations

ADPR adenosine diphosphate ribose
araf-NAD+ 2’-deoxy-2’-fluoro-nicotinamide arabinoside
adenine dinucleotide
ATA urintricarboxylic acid
B-Br- 8-bromo-cyclic adenosine diphosphate ribose
cADPR cAMP cAMP-dependent protein kinase

References


CD38 induced apoptosis independent of CD38 enzyme activity


