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Retinoic Acid-Induced CD38 Expression in HL-60 Myeloblastic Leukemia Cells Regulates Cell Differentiation or Viability Depending on Expression Levels

Thomas J. Lamkin,[1] Vivian Chin,[1] Susi Varvayanis,[1] James L. Smith,[1] R. Michael Sramkoski,[2] James W. Jacobberger,[2] and Andrew Yen[1]*

[1]Department of Biomedical Sciences, Cornell University, Ithaca, New York 14853
[2]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio, 44106

Abstract Retinoic acid-induced expression of the CD38 ectoenzyme receptor in HL-60 human myeloblastic leukemia cells is regulated by RARα and RXR, and enhanced or prevented cell differentiation depending on the level of expression per cell. RARα activation caused CD38 expression, as did RXR activation but not as effectively. Inhibition of MAPK signaling through MEK inhibition diminished the induced expression by both RARs and RXRs. Expression of CD38 enhanced retinoic acid-induced myeloid differentiation and G0 cell cycle arrest, but at higher expression levels, induced differentiation was blocked and retinoic acid induced a loss of cell viability instead. In the case of 1,25-dihydroxyvitamin D3, induced monocytic differentiation was also enhanced by CD38 and not enhanced by higher expression levels, but without induced loss of viability. Expression levels of CD38 thus regulated the cellular response to retinoic acid, either propelling cell differentiation or loss of viability. The cellular effects of CD38 thus depend on its expression level. J. Cell. Biochem. 97: 1328–1338, 2006.

Key words: CD38; retinoic acid; 1,25-dihydroxyvitamin D3; MAP

Retinoic acid (RA) regulates cell proliferation and differentiation as well as apoptosis in a variety of contexts, making its mechanism of action of interest. It is a developmental morphogen during embryogenesis, a necessary dietary factor for proper development in juveniles, and a cancer chemotherapeutic agent used in differentiation induction therapy [Sklan, 1987; Gudas, 1994; Mann et al., 2001]. RA and its metabolites are ligands for the RAR and RXR ligand activated transcription factors that are members of the steroid-thyroid hormone super family of receptors [Mangelsdorf et al., 1990, 1992, 1994; Mangelsdorf and Evans, 1995]. The RARs and RXRs form homo- and hetero-dimers that bind DNA at AGGTCA hexad consensus sequence half sites, but there can be significant promiscuity in binding sites that are departures from these canonical retinoic acid response elements (RAREs) [Mangelsdorf et al., 1994; Mangelsdorf and Evans, 1995]. For example, ligand activated RAR:RXR bind a 17 bp GT box in the 5′ promoter to transcriptionally activate the *blr1* gene while canonical RARE sequences in the region are non-functional [Wang and Yen, 2004], pointing to the need to verify function for putative RAREs. Binding of the ligand-activated complex to RAREs results in transcriptional regulation of targeted genes, ultimately giving rise to changes in cell proliferation, differentiation, or apoptosis. The identity of RA-responsive genes that are seminal to these cellular outcomes attributed to RA are of continuing interest.

The HL-60 human myeloblastic leukemia cell line [Collins et al., 1977; Breitman et al., 1980; Yen, 1990; review] has been one of the archetype

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*Correspondence to: Andrew Yen, Department of Biomedical Sciences, VRT T4-008, Cornell University, Ithaca, NY 14853. E-mail: ay13@cornell.edu

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in vitro models for studying the mechanism of action of RA. It was derived from a patient with French American British (FAB) classification M1 leukemia. HL-60 cells undergo myeloid differentiation in response to RA or monocytic differentiation in response to 1,25-dihydroxyvitamin D3 (VD3). It is thus a lineage uncommitted precursor cell where a program of lineage specific differentiation and G0 cell cycle arrest can be elicited. Treating HL-60 cells with RA results in a cascade of events that requires approximately 48 h, corresponding to two division cycles, to result in onset of functional differentiation characteristic of mature myeloid cells and G0 cell cycle arrest. [Yen et al., 1984, 1987a,b; Yen and Forbes, 1990]. This process segregates into two halves. The first segment primes cells to differentiate, but without lineage specificity. The second half determines the specific lineage, namely myeloid or monocytic. During this process, RA causes activation of MAPK signaling, which begins in the first segment and is necessary until the cells differentiate and G0 arrest [Yen et al., 1998, 1999, 2000; Hong et al., 2001]. Many specific protein expression changes have been identified during the priming process [Yen et al., 2004]. However, their dependence on RAR or RXR activation or MAPK signaling and their contribution to the propulsion of RA-induced differentiation and growth arrest is not known in most cases.

The *CD38* gene has an RARE in the first intron that makes its expression responsive to RA [Kishimoto et al., 1998]. CD38 is a 45 kDa ectoenzyme receptor [Lee, 2000; Deaglio et al., 2001; reviews]. It has an intracellular amine terminal type II signaling domain. The enzymatic domain converts NAD^+ to cADPR [Lee, 1999; Lee et al., 1999], which can mobilize Ca^{++} from the endoplasmic reticulum [Lee et al., 1994]. The receptor can be found in lipid rafts and signals through ERK activation [Zubiaur et al., 1999, 2002]. It also causes RAF activation [Zubiaur et al., 1997]. It is thus an activator of MAPK signaling. As such it can cause tyrosine phosphorylation of target molecules, including the cbl adaptor [Kontani et al., 1996], which functions in MAPK signaling. As a result of this signaling and consequential transcriptional regulation, CD38 can regulate cell proliferation. In 3T3 murine fibroblasts and HeLa human cervical carcinoma cells, it propels cell cycle progression [Zocchi et al., 1998]. However, in myeloid leukemia cells, it has been reported to provide a growth promoting [Konopleva et al., 1998] and a growth inhibitory [Todisco et al., 2000] signal. It also suppresses B-lymphopoiesis [Kumagai et al., 1995]. However, its expression is associated with a poor prognosis in chronic lymphocytic leukemia (CLL), a B-cell leukemia [D'Arena et al., 2001; Ghia et al., 2003]. In contrast, in thymic T cells, CD38 causes apoptosis [Tenca et al., 2003]. Both positive and negative growth regulatory roles have thus been attributed to CD38. The cause of these apparently divergent roles is enigmatic. This enigma hampers attempts to evaluate CD38 as a prognostic indicator in hematological neoplasias.

MATERIALS AND METHODS

Cell Lines

HL-60 cells were maintained in constant exponential growth as previously described [Brooks et al., 1996]. The cells were cultured in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 5% or when noted 10% fetal calf serum (Intergen, Co., Purchase, NY). Viability assessed by exclusion of 0.1% trypan blue was routinely in excess of 95% except as noted in certain experimental conditions. Replicate experimental cultures were initiated at a starting cell density of 0.2×10^6 cells/ml.

Chemicals

RA, RARα ligand, AM580, and pan-RXR ligand, Ro-25-6603, were used at final concentrations of 1 μM while VD3 was used at 0.5 μM (Biomol, Plymouth Meeting, PA) as previously described [Brooks et al., 1996]. Mitomycin C (Sigma-Aldrich) was used at indicated nM final concentration. PD98059 (Cell Signaling Technology, Inc., Beverly, MA) was used at 2.0 μM. For PD98059 treatment, 2.0 μM PD98059 (final added concentration) was added to cultures initiated at 0.5×10^6 cells/ml 16 h before RA-addition (−16 h), at re-suspension to 0.2×10^6 cells/ml and addition of RA (0 h) and again 16 h after RA treatment (+ 16 h).

Northern Blots and Differential Display

Eight micrograms or 48×10^6 cells of total RNA from a RNeasy or Oligotex mini-column (Qiagen, Valencia, CA) preparation was used. Differential Display (DD-PCR) [Liang and

Pardee, 1992; Liang et al., 1993] was performed using the Message Clean and RNAimage Kits (GenHunter Co., Nashville, TN) with S^{35}-labeling. To decrease false positives, replicate cultures were used to generate duplicate RNA isolates from which duplicate DD-PCR samples were run for each untreated, RA- or VD3-treated experimental condition. Bands that appeared differentially in both replicate lanes were excised, re-amplified, HindIII digested, cloned into a pGEM-Teasy vector (Promega Corporation, Madison, WI), and sequenced with T7f and S6r primers (Cornell Bio Resource Center, Ithaca, NY). Only bands consistently in both duplicate lanes were analyzed. The HT11G and HAP-10 primers revealed a large 256-bp fragment present in RA- and VD3-treated cells that was absent in untreated cells. The fragments were excised from the gel, cloned into the pGEM-Teasy vector and sequenced, and found to exactly match the human CD38 exon 8 using the GenBank data base and NCBI-BLAST program. Expression was confirmed by Northern blot on 6% formaldehyde/1% agarose gels probed with P^{32}-radiolabeled exon 8 cDNA. Equal loading was confirmed by ethidium bromide staining or EF1α cDNA probing. Typical blot results shown.

Vectors and Cloning

The pEIE expression vector, consisting of an EF1α promoter, a multiple cloning site (with NcoI, PstI, XhoI, SacI, SalI, BamHI restriction sites) incorporating a Kozak sequence, an IRES2 sequence, Enhanced Green Florescent Protein (EGFP), and neomycin gene cassette, was constructed and used for ectopic expression of CD38. Full length CD38 cDNA was XhoI-digested and gel-purified from the CDM8 vector. PCR amplification used forward primer: 5′-dCCC CCA CCA TGG ATT ACA AGG ATG ACG ACG ATA AGA TGG CCA ACT GCG AGT-3′ and reverse primer: 5-dCAT TGA AAG CCT ATG GCC AAC TGC GAG TTC AGC CCG-3′ (Invitrogen, Carlsbad, CA). Fifty microliter reactions contained 0.25 μM of each primer, 25 ng cDNA, 1 nM MgSO4, 1 × X *Pfx* reaction buffer, 0.3 mM dNTP each, and 2 U of Platinum *Pfx* DNA polymerase. Thermo-cycling consisted of 95°C/1 min, followed by 30 cycles of 95°C/20 s/55°C/30 s/68°C/90 s with a final 5 min extension. A single 1,250-bp band was gel purified. pEIE and CD38 PCR products were NcoI and HindIII-digested, gel-purified, and annealed using T4 DNA ligase (Invitrogen). Twice sequence verified clones were an exact match to the published sequence [Nata et al., 1997].

Tranfection

Fifty micrograms of plasmid was transfected into 4×10^6 exponentially growing HL-60 cells as previously described [Wightman et al., 2002]. G418 selection for 10–20 days resulted in heterogeneous cell populations with high variability in cellular expression of EGFP and CD38 protein. Stable transfectants were sorted to enrich the population for cells with transfected expression by flow cytometry using 488 nm excitation (FACS Calibur flow cytometer, BD Biosciences, San Jose, CA). Maximum autoflorescence peaks of non-transfected negative controls were aligned at $10^{0.75}$ log florescent units. Stable transfectants were sorted with identical settings. Cells expressing $10^{1.5}$ log units of florescence intensities or higher were collected. Two sequential sorts resulted in 100% EGFP-positive populations. CD38 expression was confirmed by FITC-CD38 staining or Western blot analysis using a primary anti-FLAG M2 antibody (Stratagene).

Immunoflorescence

To measure CD38 protein, 10^6 methanol-fixed cells were washed (PBS/5%FBS/0.2%NaAzide) and incubated for 1 h, 20C, with CD38 (AT1) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Methanol fixation eliminates the strong EGFP florescence in EGFP transfected cells. Following incubation and three washes, cells were incubated for 45′ in the dark at 20°C with GAM-FITC IgG_1 (Roche Diagnostics Corp., Indianapolis, IN) and then washed three times. Negative (lacking primary ab) and positive (72 h RA-treated cells) controls were included. Flow cytometric analysis was done on an EPICS 753 flow cytometer, (Beckman Coulter, Miami, FL) using 100 mW of 488 nm excitation from an argon ion laser and emitted florescence measured through 550 nm long-pass dichroic and 525 nm band-pass filters. A FALS trigger discriminated cellular signal from debris. Analysis was restricted to single cells by forward angle light scattering (FALS) and wide angle light scattering (WALS) gating. Ten thousand cells were recorded per sample.

Proliferation and Differentiation Assays and Western Blots

Proliferation measured by cell density, cell cycle arrest measured by flow cytometry of DNA stained nuclei, differentiation measured by the functional differentiation marker, inducible oxidative metabolism, and apoptosis were assessed as described previously [Brooks et al., 1996; Shah et al., 1996; Yen et al., 2001]. Antibodies against phospho-ERK1/2, PARP (cat.# 9101, 9542, Cell Signaling Technology, Inc.) and M2-FLAG (Stratagene, La Jolla, CA) were used as instructed by the manufacturers. HRP-linked anti-rabbit and anti-mouse IgG secondary antibodies (Cell Signal Technology, # 7074 and #7076) were used at 1:2,000 dilutions. SDS–PAGE was carried out as previously described [Wightman et al., 2002] using 12% gels with 0.5×10^6 cells/lane. ECL was done according to the manufacturer's instructions. Typical blots shown.

Statistical Analysis

Differential responses were determined by the mean rank tests (Cornell Department of Biostatistics technical bulletin BU-1655-M). Mean values of four independent measurements from each flask were collected from three independent experiments ($N = 3$). Error bars show ± 1 standard deviation from triplicate experiments.

RESULTS

To search for RA- or VD3-induced mRNA in HL-60 cells, differential display was performed. Replicate samples from replicate cultures of untreated and treated cells showed induced expression of a 256 bp fragment revealed by the HT11G and HAP-10 primers. The cloned fragment was sequenced and identified as the CD38 exon 8 by comparison to the GenBank sequence. Northern analysis was performed to show that the CD38 mRNA was differentially expressed. Total RNA was isolated from cultures treated or untreated with RA for 12 h and subject to Northern analysis. Figure 1A shows the blot. The CD38 mRNA expression was high in RA-treated cells and more weakly induced in VD3-treated cells. No expression was detected in untreated control cells. A Northern analysis of poly A mRNA isolated from cells after 6.5, 12, 24, 72, and 96 h of RA treatment showed that the CD38 poly A-mRNA transcript was detectable

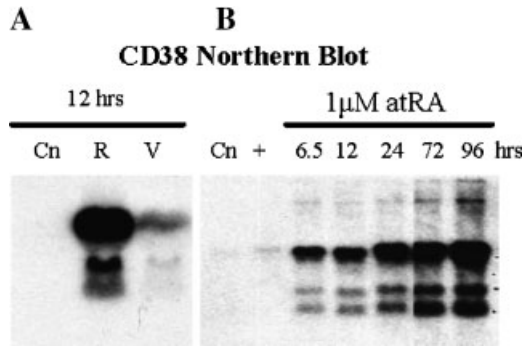

Fig. 1. Induced CD38 Expression. **A**: Shows the Northern blot for CD38 mRNA expression in cells that were untreated (Cn), 1 μM RA (R) or 0.5 μM VD3 (V) treated for 12 h. **B**: Shows the Northern blot for CD38 expression in poly A mRNA from cells that were untreated (Cn) or treated with RA for the indicated times, 6.5, 12, 24, 72, and 96 h. Weak expression in CD38 transfectants prior to enrichment by cell sorting, described later, is observed (+) whereas there is no expression in control (Cn) cells.

by 6.5 h (Fig. 1B). The amount progressively increased over time to 96 h when the population is largely differentiated and growth arrested. Multiple transcripts were observed consistent with a previous report [Nata et al., 1997]. The *CD38* gene, which consists of eight exons, has a known alternative splice transcript lacking exon 3.

To verify that RA induced CD38 protein expression consistent with mRNA expression in HL-60 cells, flow cytometry was performed. HL-60 cells were treated with RA, harvested at successive times, immunoflorescently stained with FITC-conjugated anti-CD38, and analyzed by flow cytometry. The percentage of CD38-positive cells increased after approximately 10 h and was close to maximal after approximately 16 h. Figure 2A shows the percentages as a function of time of exposure to RA. Untreated cells showed no expression. The course of appearance of the membrane-localized receptor was consistent with the course of induced mRNA expression.

To determine the dependence of RA-induced CD38 protein expression on RAR and RXR, cells were treated with either RA, an RARα-selective retinoid ligand, an RXR-selective retinoid, or both the RARα- and RXR-selective ligands together. The treated cells were harvested for analysis of membrane CD38 expression by flow cytometry after 16 h when RA-induced CD38 expression is approaching maximal. Figure 2B shows the results. RARα activation resulted in

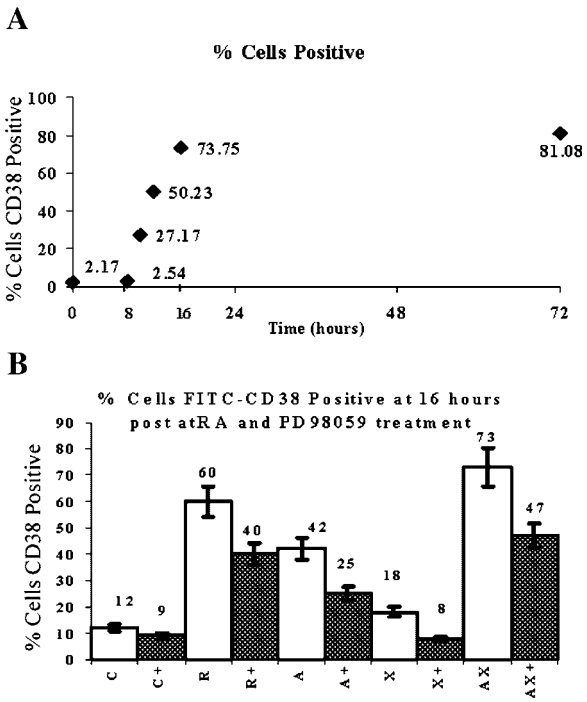

Fig. 2. Cytometric Analysis of CD38 Surface Expression. **A**: The percentage of cells positive for CD38 membrane protein as a function of time after RA treatment measured by flow cytometry. **B**: The percentage of cells positive for CD38 membrane protein for populations that were (white bars left to right): untreated; RA treated; RARα selective ligand, 1 μM AM580, treated; RXR selective ligand, 1 μM Ro 25-6603, treated; RARα selective ligand plus RXR selective ligand treated. The percentage of CD38 positive cells for the same cases for cells that were also treated with PD98059 to inhibit MAPK signaling as described in Materials and Methods is shown (dark bar to right of white bar) paired with the PD98059 untreated case (white bar). Mean values of four independent measurements from each flask were collected from three independent experiments (N = 3) with error bars showing ± 1 standard deviation from triplicate experiments.

approximately three-fourths of the expression induced by RA. RXR activation resulted in only approximately one-fourth of the RA-induced expression. But the combination of RARα plus RXR activation by the two separate ligands caused expression comparable to that seen with RA. To determine the extent to which the induced expression in each of these cases depended on MAPK signaling, the cells in each case were treated with the MEK inhibitor, PD98059. This treatment was previously shown to inhibit ERK and RAF activation caused by RA in HL-60 cells without compromising cell viability or growth rate [Yen et al., 1998]. In each case, inhibiting MAPK signaling caused a reduction of induced CD38 expression, but most of the induced response survived. CD38 expression could thus be induced by RARα activation and to a lesser extent by RXR activation. Maximal activation occurred with activation of both RARα plus RXR. Induced CD38 expression was enhanced by MAPK signaling, but not wholly dependent on it since inhibiting MAPK signaling caused only a partial reduction in induced expression in all cases.

To determine if the RA-induced CD38 expression has functional significance for the RA-induced program of cell differentiation and G0 cell cycle arrest, HL-60 cells were transfected with a bicistronic expression vector, pEIE, that expresses FLAG-tagged CD38 and EGFP from a single transcript incorporating an IRES element. The EGFP enables the isolation by florescence-activated cell sorting of a population of stably transfected cells that are totally positive for expression. Western analysis shows that successive rounds of cell sorting resulted in progressively more CD38 expression. Figure 3 shows the blot. Strong expression is seen in cells that were 100% positive for expression. To assess the amount of CD38 expression per cell, flow cytometry was used to analyze the CD38 transfectants that were RA-untreated and treated compared to vector control cells that were RA-untreated and treated. RA treatment was for 72 h to induce maximal CD38 expression. Figure 3 shows CD38 expression levels per cell in arbitrary units of florescence intensity.

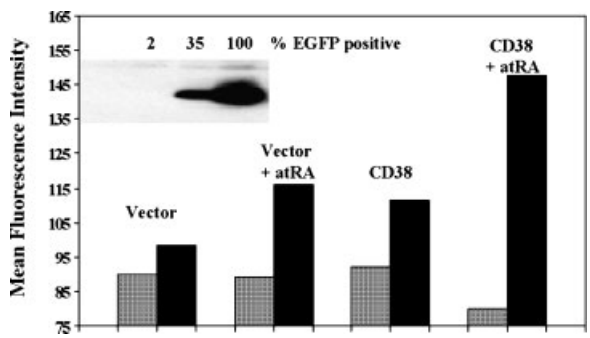

Fig. 3. CD38 Expression in Transfectants. (Insert) Western blot of CD38 expression in cells transfected with CD38 prior to florescence activated sorting for cells with positive expression and after successive cycles of sorting and amplification to enrich the population for positive cells until all cells were positive (100% positive). These cells were used for the analysis shown in the main panel: CD38 expression per cell measured by flow cytometry for (dark bars left to right) vector control, vector control treated with RA, CD38 stable transfectants after sorting, sorted CD38 transfectants treated with RA. No primary antibody controls shown by associated gray bar in each case. Coefficient of variation for flow cytometric calibration measurements (CV% = 100 ± SD/mean) was 1.5% or less.

The CD38 expression level per cell was increased in CD38 stable transfectants. The expression level per cell was comparable to that induced by RA in the vector control cells. RA treatment of the CD38 stable transfectants further increased CD38 expression levels per cell, indicating that RA-induced expression of the endogenous gene remained intact after transfection. A population of 100% CD38 transfected cells expressing CD38 at levels comparable to the maximum achieved by RA in vector control cells had thus been derived.

To determine the effect of CD38 expression on RA-induced differentiation and G0 cell cycle arrest, RA-untreated and treated vector control cells and CD38 transfectants were compared. Vector control and CD38 transfectants were cultured in the absence or presence of RA and harvested at progressive times for analysis. Functional differentiation was measured by inducible oxidative metabolism, a functional differentiation marker for terminally differentiated mature myeloid HL-60 cells. Production of cellular superoxide is detected by the reduction of nitroblue tetrazolium to formazan. By 48 h of RA treatment, the CD38-transfected cells showed enhanced differentiation compared to the vector control cells (Fig. 4C). The same measurement after 72 h showed enhanced differentiation for the CD38 transfectants also. Cell cycle arrest was measured using flow cytometry to analyze nuclear DNA content using propidium iodide staining. G1/0 cell cycle arrest is betrayed by the enrichment in the percentage of cells with G1 DNA content. Consistent with their enhanced differentiation, the CD38 transfectants showed an enhanced number of cells in G1/0 by 48 h of RA treatment compared to vector controls (Fig. 4B). After 72 h of RA treatment, the RA-treated CD38 transfectants again had more G1/0 arrested cells than the vector controls, consistent with the 48-h measurement. The enhanced cell cycle arrest is consistent with measurements of cell population density. The CD38 transfectants had a lower cell population density than the vector control cell population after 48 h and 72 h of RA treatment; although for untreated cells, the CD38 transfectants actually grew slightly faster than the vector controls (Fig. 4A). This confirms the cell cycle measurements indicating that CD38 expression enhances RA-induced G0 specific growth arrest. Expression of CD38 thus enhanced RA-induced functional differentiation and G0 arrest. Interestingly, when untreated vector control cells and CD38 transfectants were compared for susceptibility to apoptosis when challenged with progressively increasing doses of Mitomycin C (MMC), the cells were very similar; however, the CD38 transfectants showed slightly more apoptotic cells discerned by the nuclear morphology of Hoechst/propidium iodide stained cells as previously reported [Yen et al., 2001] for a number of increasing MMC concentrations (Fig. 5A). PARP cleavage was used to verify the dose-dependent MMC-induced apoptosis (Fig. 5B).

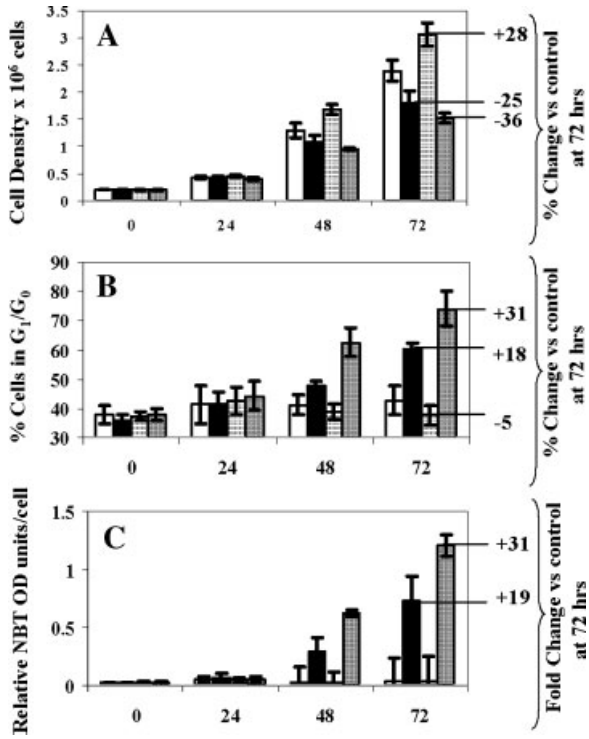

Fig. 4. Effect of RA on Differentiation and Proliferation of CD38 Transfectants. Cell population density (**A**); percentage of cells in G1/0 measured by flow cytometry (**B**); inducible oxidative metabolism, a functional differentiation marker, colorimetrically measured by reduction of nitroblue tetrazolium, NBT (**C**); for (left to right columns) untreated control (white), RA-treated vector control (black), untreated CD38 transfectants (light gray), RA-treated CD38 transfectants (dark gray). Statistics and replicates as described in Figure 1 legend.

To determine if different CD38 expression levels per cell might influence their response to RA, the CD38 transfectants were sorted by florescence-activated cell sorting based on EGFP expressed from the same transcript as the CD38. Sorted populations were chosen to have approximately median or one log higher

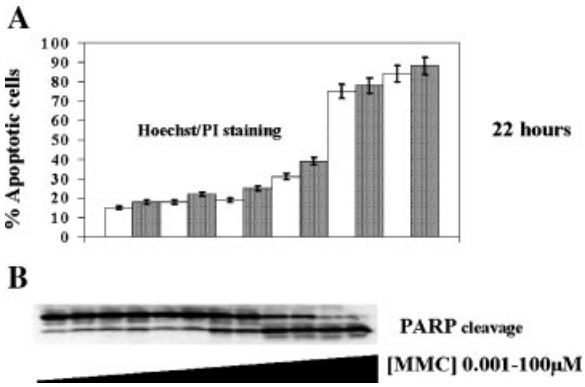

Fig. 5. Response to apoptotic challenge by treating with increasing concentrations of mitomycin C (0.001–100 nM) for 22 h. detected cytologically by Hoechst/propidium iodide staining (**A**) and PARP cleavage (**B**) for vector control cells (white) and CD38 transfectants (shaded). After 72 h of RA treatment, the functional differentiation marker was increased 19-fold in vector control cells versus 31-fold in CD38 transfectants. The percentage of cells in G1/0 increased by 18% for vector control and 31% for CD38 transfectants from the approximately 40% typically in untreated exponentially proliferating cells.

expression. This isolated cell populations with expression in the middle, called medium expressors, and also at the high extreme, called high expressors, of the range observed. Figure 6 shows the flow cytometric histograms of the original and sorted populations. To verify the functional signaling activity of the CD38 receptor in medium and high expressors, ERK activation, which is a known CD38 signaling route, was measured. Figure 7 shows ERK activation for the vector control, medium expressors and high expressors stimulated with serum in the absence or presence of RA. Cells were cultured with low serum (5%) or high serum (10%) in the absence or presence of 1 μM RA for 24 h. The transfectants showed greater ERK activation in response to increasing serum for both untreated and RA-treated cells. In response to serum plus RA, ERK activation was higher in high expressors than medium expressors as anticipated. As previously reported for wt HL-60 [Yen et al., 1998, 2000; Hong et al., 2001], in vector-control cells RA increased the amount of activated ERK, which was also

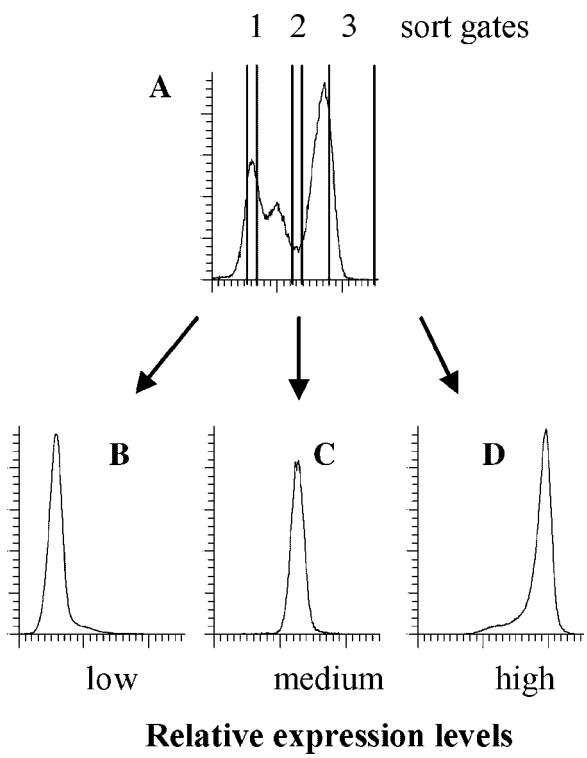

Fig. 6. Flow Cytometric Histograms of CD38 Expression per Cell for the CD38 Transfectants (described in Fig. 2, where all cells are CD38 positive) and Sublines derived from it by Florescence activated cell sorting. Sort gates (**A**) set on the CD38 transfected cells were chosen to isolate sublines with low, middle, and high expression. Flow cytometric analysis of the sorted populations verifying expression levels in derived sublines (**B**, **C**, **D**). The middle expressors, having approximately median expression levels per cell, and the high expressors, having almost one log higher expression per cell, were subsequently used.

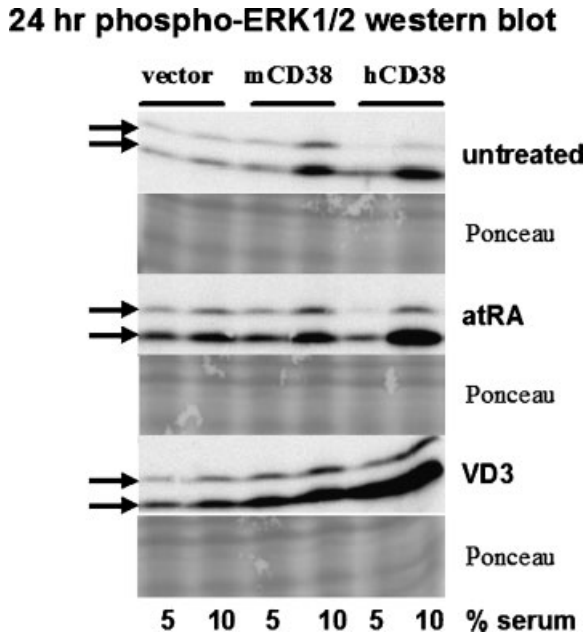

Fig. 7. Phospho-ERK Western Blot. Vector control (vector), medium CD38 expressors (mCD38), and high CD38 expressors were either untreated or cultured with 1 μM RA (atRA), 0.5 μM 1,25-dihydroxy vitamin D3 (VD3) with low (5%) or high (10%) serum for 24 h. Uniform lane loading and electro-transfer is verified in the Ponceau S stained membranes.

enhanced by serum. The ectopic CD38 thus appears to be signaling.

These medium- and high-expressor sublines were used to determine if CD38 expression levels affected RA-induced cellular differentiation. Vector control, medium CD38 expressors, and high CD38 expressors were treated or not with RA or VD3. Functional differentiation, cell population viability, and cell population density were measured at sequential times after initiation of cultures. As before, functional differentiation was measured by inducible oxidative metabolism, and viability was assessed with trypan blue exclusion. Figure 8 shows the results. RA induced differentiation of vector control cells (Fig. 8G) and the differentiation was enhanced in medium expressors (Fig. 8H). However, differentiation was not enhanced but reduced in high expressors (Fig. 8I). In vector control or medium expressor cells that were untreated or RA-treated, viability remained high at 95–100% (Fig. 8D,E, vector control and medium expressors, respectively). But in high expressors, RA caused a precipitous drop in cell viability to approximately 50% by 96 h, whereas viability of the untreated cells remained high (Fig. 8F). In high CD38 expressors, RA-induced differentiation was thus reduced, and RA caused a loss of viability/apoptosis instead. The cell population density reflecting the growth of these different cell populations is consistent with these effects. Growth of vector control cells was slowed by RA (Fig. 8A) as expected, and growth was more inhibited by RA in medium expressors (Fig. 8B). This is consistent with the enhanced differentiation of medium expressors. In high expressors, growth of the RA-treated population was more grossly inhibited consistent with the loss of viability/apoptosis (Fig. 8C). Consistent with the above cellular effects of CD38, RA-induced hypophosphorylation of the RB protein, typically associated with differentiation and G0 arrest in HL-60 cells [Brooks et al., 1996], was enhanced for medium expressors, but blocked by high CD38 expression (data not shown). Cellular response to RA thus depends on the expression level of CD38. While medium CD38 expressors had enhanced RA-induced differentiation, high CD38 expression was inhibitory for RA-induced differentiation. In sum, medium CD38 expression enhanced differentiation, but high CD38 expression did not and resulted in loss of cell viability instead. CD38 thus regulates cellular outcomes in response to RA depending on its expression level per cell. The case for VD3 is similar except that the high CD38 expression did not suppress VD3-induced differentiation as strongly and there was no VD3-induced loss of viability in high expressors, indicating that the high CD38 expression by itself did not cause loss of viability for these differentiating cells.

DISCUSSION

The present results show that RA induced the expression of the CD38 mRNA and protein, consistent with earlier reports that the CD38 first intron contained an RARE and that CD38 protein expression was RA-inducible [Kishimoto et al., 1998]. The present studies show that RARα activation induced CD38 protein expression, as did RXR activation, but less effectively. Maximal CD38 expression, comparable to that induced by RA, was achieved by RARα plus RXR activation. Both RARα- and RXR-induced CD38 expression was augmented by MAPK signaling. Thus, maximal-induced expression of CD38 utilized RARα, RXR, and MAPK signaling. CD38 expression

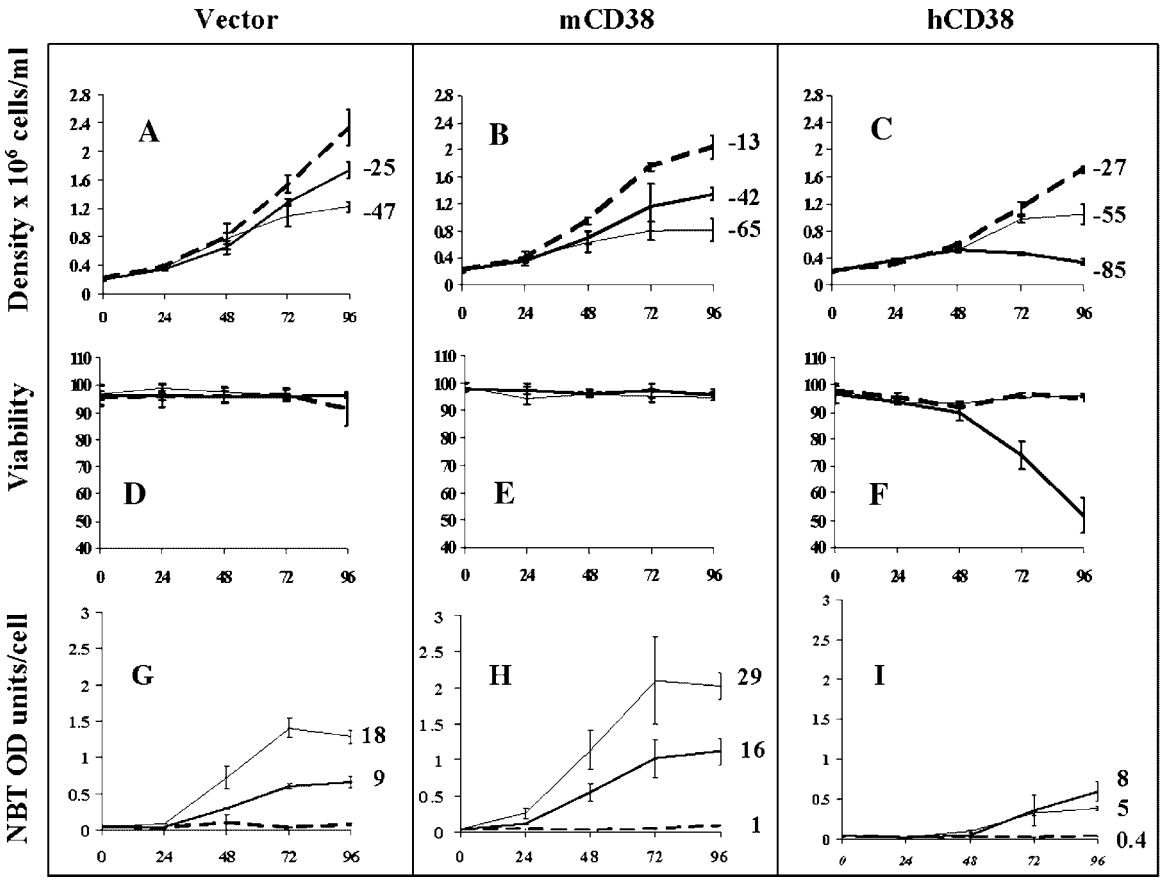

Fig. 8. Growth and differentiation of RA- and VD3-treated vector control, medium CD38 expressors and high CD38 expressors. Cell population density (**A**, **B**, **C**), viability (**D**, **E**, **F**), and functional differentiation (**G**, **H**, **I**) for vector control cells (vector) (A, D, G), medium CD38 expressors (mCD38) (B, E, H), and high CD38 expressors (hCD38) (C, F, I) that were untreated (dashed line), treated with RA (thick line) or treated with VD3 (thin line). Numbers to the right of the graphs represent change relative to untreated vector control at 96 h. For example, RA caused a 25% reduction in cell density in vector controls compared to a 42% reduction in mCD38. RA caused an 18-fold increase in oxidative metabolism for vector controls compared to a 29-fold increase in mCD38 cells but only a fivefold change in hCD38 cells.

contributes to propulsion of RA-induced differentiation and growth arrest, attributed to G0 cell cycle arrest. Overexpression of CD38 enhanced RA-induced functional differentiation and G0 cell cycle arrest. The effect, however, depended on the CD38 expression level per cell. High CD38 expression levels per cell were inhibitory for RA-induced myeloid differentiation, and RA induced a loss of viability instead. High CD38 expression was not as inhibitory for VD3-induced monocytic differentiation, and there was no loss of viability like for RA. This may indicate differences in how the RA-elicited myeloid differentiation pathway versus the VD3-elicited monocytic differentiation pathway utilize CD38. In summary, RA caused CD38 mRNA and protein expression through RAR and RXR activation and MAPK signaling, and CD38 expression was either a positive or negative regulator of induced cell differentiation and growth arrest, depending on the expression level per cell.

CD38 has been attributed with different effects on cell growth. In some cases, such as 3T3 or HeLa cells, it promotes cell cycle progression [Zocchi et al., 1998]. In other cases, such as B lymphocytes [Kumagai et al., 1995] or T lymphocytes [Tenca et al., 2003], it is growth inhibitory and can cause apoptosis. However, in CLL, a B cell leukemia, CD38 is an indicator of poor prognosis [D'Arena et al., 2001; Ghia et al., 2003]. Even in the same type cells, namely myeloid leukemia cells, it has been reported to have growth promoting or inhibitory apoptotic effects [Konopleva et al., 1998; Todisco et al., 2000]. Its cellular effects have thus been somewhat enigmatic. The present results suggest that this enigma may in part represent the

potentially different effects of CD38 at different expression levels. The cellular outcome thus depends on the dosage of CD38 signaling per cell. The present studies suggest that further analysis of the effects of CD38 should take into account the expression level of the receptor per cell. In this vein, studies of the value of CD38 as a prognostic indicator in certain leukemias may be more informative if not only the number of positive cells, but the CD38 expression level per cell are considered in the analysis.

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