

Ligation of CD38 Suppresses Human B Lymphopoiesis

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Summary

CD38 is a transmembrane glycoprotein expressed in many cell types, including lymphoid progenitors and activated lymphocytes. High levels of CD38 expression on immature lymphoid cells suggest its role in the regulation of cell growth and differentiation, but there is no evidence demonstrating a functional activity of CD38 on these cells. We used stroma-supported cultures of B cell progenitors and anti-CD38 monoclonal antibodies (T16 and IB4) to study CD38 function. In cultures of normal bone marrow CD19⁺ cells ($n = 5$), addition of anti-CD38 markedly reduced the number of cells recovered after 7 d. Cell loss was greatest among CD19⁺sIg⁻ B cell progenitors (mean cell recovery \pm SD = $7.2 \pm 11.7\%$ of recovery in control cultures) and extended to CD19⁺CD34⁺ B cells (the most immature subset; $7.6 \pm 2.2\%$). In contrast, CD38 ligation did not substantially affect cell numbers in cultures of normal peripheral blood or tonsillar B cells. In stroma-supported cultures of 22 B-lineage acute lymphoblastic leukemia cases, anti-CD38 suppressed recovery of CD19⁺sIg⁻ leukemic cells. CD38 ligation also suppressed the growth of immature lymphoid cell lines cultured on stroma and, in some cases, in the presence of stroma-derived cytokines (interleukin [IL] 7, IL-3, and/or stem cell factor), but did not inhibit growth in stroma- or cytokine- free cultures. DNA content and DNA fragmentation studies showed that CD38 ligation of stroma-supported cells resulted in both inhibition of DNA synthesis and induction of apoptosis. It is known that CD38 catalyzes nicotinamide adenine dinucleotide (NAD⁺) hydrolysis into cyclic ADP-ribose (cADPR) and ADPR. However, no changes in NAD⁺ hydrolysis or cADPR and ADPR production after CD38 ligation were found by high-performance liquid chromatography; addition of NAD⁺, ADPR, or cADPR to cultures of lymphoid progenitors did not offset the inhibitory effects of anti-CD38. Thus, anti-CD38 does not suppress B lymphopoiesis by altering the enzymatic function of the molecule. In conclusion, these data show that CD38 ligation inhibits the growth of immature B lymphoid cells in the bone marrow microenvironment, and suggest that CD38 interaction with a putative ligand represents a novel regulatory mechanism of B lymphopoiesis.

CD38, a 45-kD transmembrane glycoprotein, is highly expressed on the surface of lymphoid, myeloid, and erythroid progenitor cells, activated T and B lymphocytes, NK cells, and plasma cells (1-6). In most cases of acute lymphoblastic leukemia (ALL)¹, leukemic cells—the malignant counterparts of immature lymphoid cells—are also CD38⁺ (1, 3, 4).

Little is known about the biologic function and physiologic role of CD38. Ligation of CD38 with specific antibodies has a stimulatory effect on cultured lymphocytes (7), and it enhances DNA synthesis in the presence of other stimuli such as CD3, IL-4, and IL-2 plus accessory cells (7-9). Engagement of CD38 also rescues germinal center cells from apoptosis (10) and impairs lymphocyte adhesion to endothelial cells (11). Comodulation experiments have shown that CD38 is physically associated with CD3 on T cells, with surface Ig, CD19, and CD21 on B cells, and with CD16 on NK cells (12). Recently, CD38 has been found to be structurally homologous to *Aplysia californica* ADP-ribosyl cyclase (13), an enzyme that catalyzes the conversion of nicotinamide ade-

¹ Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; cADPR, cyclic ADP-ribose; NAD⁺, nicotinamide adenine dinucleotide; PBSA, PBS containing 0.2% BSA and 0.2% sodium azide; SCF, stem cell factor; sIg, surface Ig.

nine dinucleotide (NAD⁺) to cyclic ADP-ribose (cADPR), a calcium-mobilizing agent (14). Indeed, CD38 has such enzymatic activity (9, 15–19), suggesting that it may exert its effects on lymphocyte function through ADPR cyclase activity, although the precise mechanisms involved are unclear (9).

The strong expression of CD38 on highly proliferating progenitor cells in the bone marrow implies a possible role of this molecule in the regulation of growth and differentiation of lymphoid precursors; however, there is no evidence to date of CD38's functional activity on these cells. Human immature B cells rapidly die in vitro unless they are supported by bone marrow-derived stromal cells (20). The establishment of tissue culture methods that prevent apoptotic cell death and sustain the growth of normal and leukemic immature B cells has opened the opportunity to investigate the molecules that influence human B lymphopoiesis (20–23). In this study, we used anti-CD38 mAbs and stroma-supported cultures of B cell progenitors to investigate the function of CD38 during normal and leukemic B cell differentiation and found that ligation of CD38 suppresses survival and growth of normal and leukemic immature B cells.

Materials and Methods

Cells. Bone marrow samples were taken, with informed consent and Institutional Review Board approval, from 5 healthy bone marrow transplant donors, aged 2–30 yr (median, 10 yr), and from 22 patients with newly diagnosed B-lineage ALL, aged 1–18 yr (median, 5 yr). Diagnosis was unequivocal by morphologic, cytochemical, and immunophenotypic criteria. In each case of ALL, >80% of the blasts were positive for CD19, CD22, terminal deoxynucleotidyl transferase, and MHC-class II antigens, and negative for surface Ig. Peripheral blood samples were obtained from healthy adult donors. Tonsils were obtained from routine tonsillectomies; cell suspensions were prepared by use of forceps and surgical blades. Mononuclear cells were collected after centrifugation on a density gradient (Lymphoprep; Nycomed, Oslo, Norway) and washed three times in PBS and once in AIM-V medium (Gibco Laboratories, Grand Island, NY). Normal CD19⁺ bone marrow and peripheral blood cells were purified by use of CD19-immunomagnetic beads (Dyna, Oslo, Norway). Cells were detached from the beads by use of a goat antiserum to mouse Ig Fab (DETACHaBEAD; Dynal). At the end of the procedure, 90–98% of cells were CD19⁺. T cells were removed from suspensions of tonsillar lymphocytes by use of a mixture of magnetic beads conjugated to CD4 and CD8 antibodies (Dyna). Cell-sorting procedures were performed according to the manufacturer's instructions. Immature B lineage ALL (RS4;11, OP-1, 380, REH, 697, NALM6), Burkitt's lymphoma (RAJI) and T-lineage ALL (MOLT4, CEM-C7) cell lines were obtained from our cell bank; the EBV-transformed B lymphoblastoid cell line J.R. was obtained by EBV infection of peripheral blood B lymphocytes from a healthy individual. All cell lines were maintained in RPMI-1640 (Whittaker Bioproducts Inc., Walkersville, MD) with 10% FCS (Whittaker Bioproducts Inc.), L-glutamine, and antibiotics.

To obtain bone marrow stromal cells, we collected mononuclear cells from normal marrow donors. The cells were separated as described above and washed three times in RPMI-1640. Stromal layers were prepared in flat-bottomed 96- or 24-well plates (Costar Corp., Cambridge, MA) and fed with RPMI-1640, 10% FCS, and 10⁻⁶ M hydrocortisone (20, 23–25).

Cell Culture Studies. Before each experiment, we removed the media from cultures of stromal cells and washed the adherent cells seven times with RPMI-1640 to fully remove hydrocortisone (20, 23–25). Normal and leukemic cells were plated in culture within 5 h of collection. The cells' viability consistently exceeded 90% by trypan blue dye exclusion. Leukemic and normal cells were resuspended in serum-free AIM-V medium, and cell lines were resuspended in RPMI-1640 plus 10% FCS. 200 μ l of the cell suspension (0.1–1.5 cells \times 10⁶/ml) were then placed in the wells of a 96-well flat-bottomed tissue culture plate or seeded onto marrow stromal cells in an identical plate. In some experiments, cells were cultured separated from stromal layers by use of a 0.4- μ m microporous membrane (Transwell; Costar) (23). All cell cultures were incubated at 37°C in 5% CO₂ with 90% humidity. At the termination of cultures, cells were harvested by vigorous pipetting. Microscopic examination of the plates ensured that all cells in the wells (i.e., adherent and nonadherent) were recovered. Cells cultured on stroma were suspended in PBS and passed through a 19-gauge needle to disrupt clumps. At least two replicate test were performed in each experiment.

We selected two anti-CD38 mAbs for these studies: T16 of IgG1 class was purchased from AMAC (Westbrook, ME); IB4 of IgG2a class was produced in our laboratories. Both antibodies have been shown to have characteristic CD38 reactivity by both immunofluorescence and immunoprecipitation (12, 26); T16 was also shown to react with COS cells transfected with human CD38 cDNA (27). Cross-blocking experiments showed that both antibodies reacted with the same epitope (data not shown). In some experiments, F(ab')₂ fragments of IB4, produced by standard methods (28), were used. CD38 antibodies and isotype-matched unreactive controls were dialyzed in PBS, sterile filtered, and added to the cultures at 2 μ g/ml, a saturating concentration for the CD38 antibodies. Recombinant cytokines IL-3, IL-7, and stem cell factor (SCF) were purchased from R&D Systems, Inc. (Minneapolis, MN).

Flow Cytometric Analysis of Cell Numbers, Cell Cycle, and DNA Fragmentation. Viable cells in culture were enumerated by flow cytometry as previously described (20, 23–25). Briefly, harvested cells were washed three times in PBS containing 0.2% BSA and 0.2% sodium azide (PBSA). To assess cell survival, we resuspended test and control cells in equal amounts of PBSA. Aliquots of the suspensions were placed in Falcon tubes (Becton Dickinson & Co., Lincoln Park, NJ) and either washed in PBSA and fixed in 0.5% paraformaldehyde or incubated with the following antibodies: CD19 (Leu 12) conjugated to FITC, PE, or peridin chlorophyl protein (PerCP; Becton Dickinson & Co., San Jose, CA); CD34 (HPCA-2) conjugated to PE (Becton Dickinson & Co.); CD7 (Leu9) conjugated to FITC (Becton Dickinson & Co.); a mixture of goat anti-human Ig κ + λ light chain antisera conjugated to FITC (Southern Biotechnology Associates, Birmingham, AL); and/or goat anti-human IgD conjugated to FITC (Southern Biotechnology Associates, Birmingham, AL); and/or goat anti-human IgD conjugated to FITC (Southern Biotechnology Associates). Isotype-matched nonreactive antibodies were used as a control. After two washes in PBSA, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a FACScan[®] flow cytometer with Lysis II software (Becton Dickinson & Co.). We designed "gates" in the light-scattering dot-plots including the vast majority of viable lymphoid cells. Before and after culture, we counted cells with these light-scattering properties that passed through the gate within a period of time (e.g., 30 s). The results were corrected according to the distribution of cells expressing different immunophenotypes.

Expression of CD38 on various cell types was studied with anti-CD38 PE (Becton Dickinson & Co.), which reacts with the same

epitope identified by T16 and IB4 (data not shown). Cell cycle analysis was performed by labeling cells with propidium iodide and determining their DNA content by flow cytometry (FACScan[®] with CellFit software; Becton Dickinson & Co.) as described elsewhere (29). DNA fragmentation was evaluated by flow cytometry after cell permeabilization and staining with digoxigenin-dUTP, as described by Gold et al. (30).

Measurement of NAD⁺ Hydrolase and ADPR Cyclase Activity. Nicotinamide, β -NAD, and ADPR were obtained from Sigma Chemical Co. (St. Louis, MO). cADPR was obtained from Amersham International (Amersham, UK). Immature B cells (OP-1 cell line; 31) were washed three times in normal saline solution and resuspended at a concentration of 5×10^6 cells/ml. 250 μ l of the cell suspension was incubated for 30 min at 20°C with anti-CD38 (IB4) or a nonreactive antibody, after which 80 μ M NAD⁺ was added. Extracellular concentrations of NAD⁺, nicotinamide, ADPR, and cADPR were determined at different timepoints in supernatants obtained after cell centrifugation by HPLC with UV detection at 254 nm as described by Lee et al. (32), with slight modifications. We used a Partisil 5 SAX (0.46 \times 10 cm) column (Whatman Inc., Hillsboro, OR) and a linear gradient of 0.005–0.1 M ammonium phosphate monobasic in 30 min at a flow rate of 1 ml/min. The column was reequilibrated with 0.005 M ammonium phosphate for an additional 15 min before the next injection. Standards for nicotinamide, β -NAD, ADPR, and cADPR were made in double-distilled H₂O. The standard curve for nicotinamide ranged from 5 to 115 μ M; for β -NAD, from 2 to 47 μ M; for ADPR, from 3 to 54 μ M; and for cADPR, from 0.24 to 5.8 μ M. The coefficient of variation was <15%, and controls were within 10% of their true value for all experiments.

Results

Effects of CD38 Ligation in Cultures of Normal B Cell Progenitors and Mature B Cells. To investigate the effects of CD38 perturbation on the growth of normal B cell progenitors, we purified B lymphoid cells from normal bone marrow samples by use of magnetic beads conjugated to CD19 (a pan-B cell marker), seeded them onto allogeneic bone marrow stromal layers, and added CD38 antibodies (T16 or IB4) to the cultures. We used triple-color immunofluorescence and flow cytometry to quantify the effects of CD38 ligation at different stages of normal B cell differentiation. At the end of the cultures, cells were stained with antibodies to CD19, CD34, and surface Ig (sIg) simultaneously, permitting recognition of relatively immature (CD34⁺) and mature (sIg⁺) subpopulations, as well as a third group of cells (CD19⁺CD34⁻sIg⁻) at an intermediate level of differentiation. In all samples, CD34 and sIg were consistently expressed by different cell subpopulations.

In five experiments, >90% of the CD19⁺ cells were also CD38⁺ before culture (Fig. 1 A). Addition of anti-CD38 markedly reduced cell recovery after 7 d of culture, compared with control cultures with isotype-matched nonreactive antibodies (Fig. 1, B and C). Cell loss was particularly severe among CD19⁺sIg⁻ B cell progenitors (mean cell recovery \pm SD = 7.2 \pm 11.7% of recovery in control wells). The suppressive effect of CD38 ligation extended to the most immature CD19⁺sIg⁻ B lineage cells, which were identified by CD34 expression: among this cell subset, mean cell recovery

was 7.6 \pm 2.2% of control cultures (Fig. 1 C). Anti-CD38 antibodies also affected the recovery of bone marrow sIg⁺ lymphocytes, although to a lesser extent (mean cell recovery = 42.0 \pm 23.5%; p < 0.02 by t test; Fig. 1 C).

CD38 was expressed, albeit weakly, on the majority of circulating peripheral blood B lymphocytes (Fig. 1 A). In contrast to bone marrow B cells, however, recovery of these cells was not substantially reduced by CD38 ligation during culture on stromal layers. Recovery of circulating CD19⁺sIg⁺ B cells after 7 d of culture in the presence of anti-CD38 (T16 or IB4) was 107.0 \pm 13.1% of control cultures in four experiments (Fig. 1, B and C). Finally, we tested the effects of ligating CD38 in tonsillar B lymphocytes. Virtually all tonsillar CD19⁺ B cells were CD38⁺, the antigen being expressed with the highest intensity on sIgD⁻CD10⁺ cells and at the lowest intensity on sIgD⁺CD10⁻ cells, corresponding to germinal center and B corona cells, respectively (33; Fig. 1 A). Cell recovery after CD38 ligation by T16 or IB4 was heterogeneous but, overall, was not suppressed as seen with bone marrow CD19⁺ cells. In three experiments, cell recovery after 7 d of stroma coculture in the presence of anti-CD38 was 88.7 \pm 14.6% of control cultures among CD19⁺sIgD⁻ cells; within the CD19⁺sIgD⁺ subset, relative cell recovery was 91.3 \pm 18.2% (Fig. 1, B and C). Thus, ligation of CD38 markedly suppressed cell recovery of bone marrow B cells but had no consistent effect on circulating B lymphocytes and mature reactive lymphoid cells from tonsil.

Effects of CD38 Ligation in Cultures of Leukemic Lymphoid Cells and Cell Lines. The inhibitory effects of anti-CD38 antibodies were also seen in stroma-supported cultures of leukemic cells. In 22 cases of ALL, all comprising clonal expansions of CD19⁺sIg⁻ B cell precursors, CD38 was expressed in >90% of lymphoblasts. When anti-CD38 antibodies (T16 or IB4) were added to the stroma-supported cultures, the cell recovery decreased in all cases (mean cell recovery = 29.1 \pm 21.3% of control cultures).

Next, we studied CD38 function in continuously growing leukemic cell lines that are phenotypically equivalent to normal immature lymphoid cells. In the six cell lines of immature B cell origin (RS4;11, OP-1, 380, REH, 697, NALM6), anti-CD38 inhibited growth on stromal layers (range of mean cell recoveries after 4 d of culture = 7.0–32.7%; Table 1). Similar inhibitory activity was observed with CD38⁺ lines of T cell origin (MOLT4 and CEM-C7). In contrast, the suppressive effects of CD38 on the EBV-transformed (EBV J.R.) and the Burkitt's lymphoma (RAJI) cell lines (both CD38⁺) cultured on stroma were much less marked or essentially absent (mean cell recovery = 85.7% and 99.0%, respectively; Table 1).

The inhibitory effects of anti-CD38 on immature lymphoid cells were dose dependent and achieved 90% of the maximal effect at a concentration of 0.4 μ g/ml. Surprisingly, in parallel stroma-free cultures, neither of the two anti-CD38 antibodies suppressed cell growth of immature lymphoid cells (Table 1). We considered the possibility that stroma could act as a cross-linker of CD38 antibodies. However, F(ab')₂ fragments of IB4 also had an inhibitory effect on the growth of immature lymphoid cell lines, although this was less potent than that observed with the whole antibody molecule.

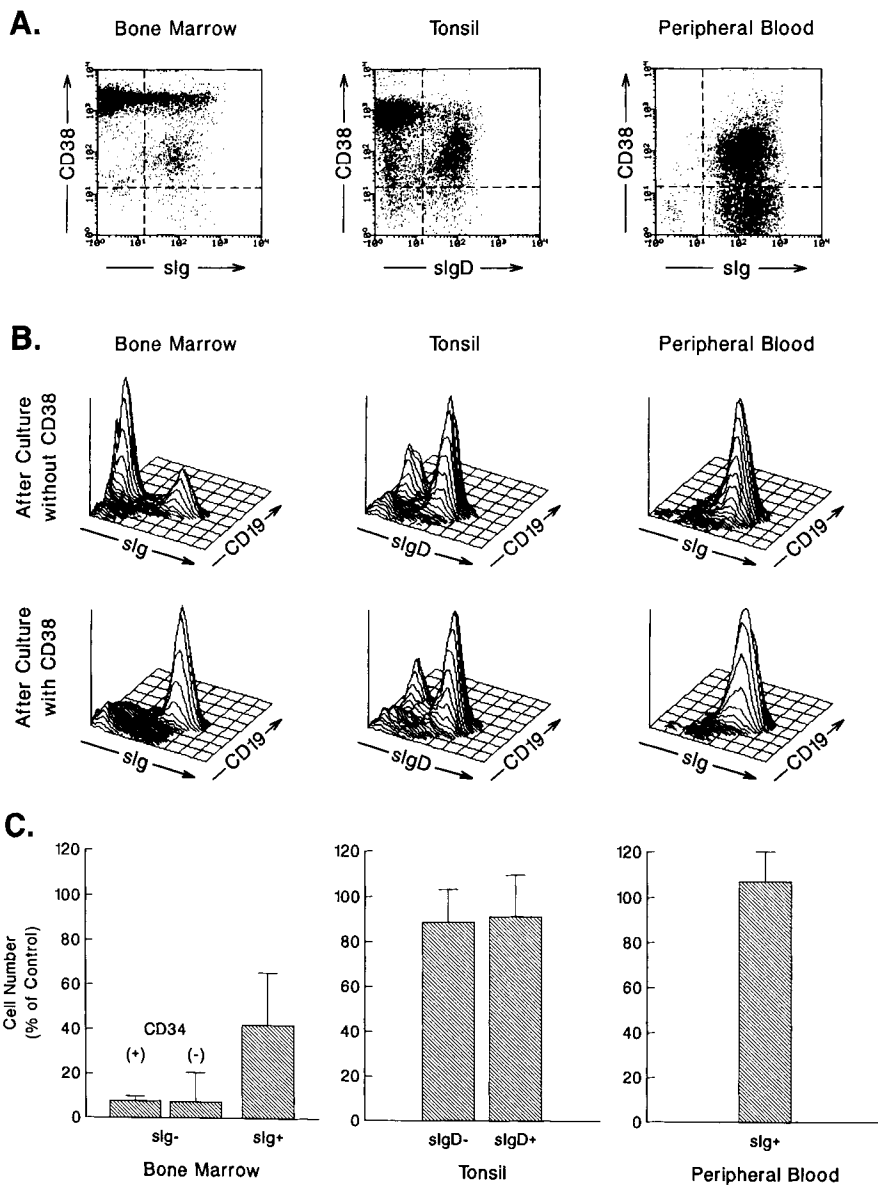


Figure 1. CD38 expression and the effect of CD38 ligation in normal B cells at different stages of differentiation. (A) Flow cytometric dot plots of CD38 expression in CD19⁺ cells from bone marrow, tonsil, and peripheral blood. In bone marrow B cells, CD38 expression is at the highest levels: sIg⁻ and a proportion of sIg⁺ cells are strongly CD38⁺. In tonsil, CD38 levels are higher among sIgD⁻ cells. PBL are either weakly CD38⁺ or CD38⁻. (B) Isometric contour plots showing flow cytometric analysis of cells labeled with the indicated antibodies. Highly enriched bone marrow, peripheral blood, and tonsillar B cells were cultured for 7 d on allogeneic bone marrow stroma with a nonreactive antibody or anti-CD38 (T16). Addition of anti-CD38 antibody markedly reduced the number of CD19⁺sIg⁻ immature bone marrow B cells recovered, but did not decrease the recovery of CD19⁺sIg⁺ peripheral blood B cells, CD19⁺sIgD⁺, or CD19⁺sIgD⁻ tonsil cells. (C) Cell recovery (mean \pm SD) of anti-CD39 treated relative to control cultures in experiments with purified B lineage cells from bone marrow ($n = 5$), tonsil ($n = 3$), and peripheral blood ($n = 4$).

After 4-d cultures with IB4 (F(ab')₂ on stroma, cell recovery relative to control cultures was 70 and 47% in OP-1 and RS4;11, respectively. Thus, the inhibitory effects of anti-CD38 antibodies in the presence of stroma do not depend on extensive antibody cross-linking. Moreover, cross-linking of anti-CD38 with a goat anti-mouse antibody in the absence of stroma did not alter cell recovery (data not shown).

To investigate whether adhesion to stroma was necessary to render cells responsive to anti-CD38, we cultured the immature B cell lines OP-1 and RS4;11 separated from stroma by a microporous membrane (Fig. 2). Separation from stroma reduced but did not abolish the inhibitory effect of anti-CD38. Thus, adhesion to stromal layers is not strictly required for anti-CD38-mediated suppression of growth, which can occur in the presence of diffusible stromal factors.

In situ staining of bone marrow stromal layers revealed no

CD38 expression on fibroblasts and low CD38 expression on occasional large, round, macrophage-like cells (data not shown). However, to test the possibility that the effects of anti-CD38 were indirectly induced by stimulation of stroma, we labeled OP-1 and RS4;11 immature B cells with anti-CD38, removed excess antibody by washing, and seeded cells onto stroma. Reduction in cell recovery was virtually identical to that of parallel experiments in which the antibody was directly added to the cultures (data not shown). We also considered the possibility that ligation of CD38 on progenitor cells could induce the production of factors which, in turn, would stimulate stromal cells to secrete inhibitory molecules. To test this possibility, we performed experiments in which one of two cell lines, CEM-C7 and 380, was individually labeled with anti-CD38 and mixed with unlabeled cells of the other line. After culture on stromal layers, cell recovery was reduced only

Table 1. Effects of CD38 Ligation on Cell Recovery of Immature Lymphoid Cell Lines

Cell line	Phenotype (CD)*	Cell recovery with anti-CD38†	
		With stroma	Without stroma
%			
B lineage ALL			
RS4;11	19 ⁺ , 34 ⁻ , 10 ⁻ , 20 ⁻	13.3 ± 7.2	96.5 ± 7.8
OP-1	19 ⁺ , 34 ⁺ , 10 ⁺ , 20 ⁻	32.7 ± 17.8	104.7 ± 4.2
380	19 ⁺ , 34 ⁻ , 10 ⁺ , 20 ⁺	7.0 ± 7.0	116.1 ± 10.9
REH	19 ⁺ , 34 ⁻ , 10 ⁺ , 20 ⁻	22.3 ± 5.5	105.3 ± 4.9
697	19 ⁺ , 34 ⁻ , 10 ⁺ , 20 ⁺	11.8 ± 2.1	102.3 ± 3.8
NALM6	19 ⁺ , 34 ⁻ , 10 ⁺ , 20 ⁻	31.3 ± 9.0	114.3 ± 12.5
T-lineage ALL			
MOLT4	7 ⁺ , 3 ⁻ , 4 ⁻ , 8 ⁺	39.8 ± 1.5	96.0 ± 1.4
CEM-C7	7 ⁺ , 3 ⁻ , 4 ⁺ , 8 ⁻	16.7 ± 0.6	97.2 ± 0.5
Burkitt's lymphoma			
RAJI	19 ⁺ , 34 ⁻ , 10 ⁺ , 20 ⁺	99.0 ± 11.5	110.5 ± 18.1
B-lymphoblastoid			
EBV J.R.	19 ⁺ , 34 ⁻ , 10 ⁻ , 20 ⁺	85.7 ± 10.7	98.2 ± 6.0

* CD numbers are indicated. All lines were CD38⁺. T lineage cell lines MOLT4 and CEM-C7 were cytoplasmic CD3⁺.

† Values represent percentage of cell recovery (mean ± SD of at least three experiments) relative to control cultures with isotype-matched nonreactive antibodies after 4 d of culture with anti-CD38 (T16 or IB4).

in the cells prelabeled with CD38 (Fig. 3). Thus, the suppressive effects of adding CD38 antibodies to cultures are due to signal transduction directly caused by CD38 ligation and are not indirect effects due to stimulation of stromal cells.

Finally, we investigated whether cells could be rendered

sensitive to anti-CD38 by stroma-derived cytokines instead of stromal layers. The immature B cell lines RS4;11, OP-1, 380, REH, 697, and NALM6 were incubated with IL-7 (25 ng/ml), IL-3 (10 ng/ml), and/or SCF (20 ng/ml), with and

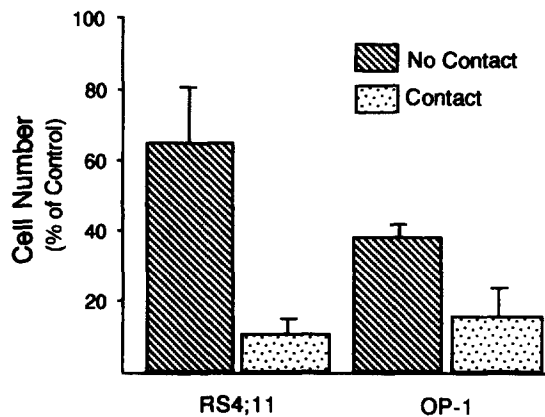


Figure 2. Suppression of cell growth by CD38 ligation is greater when immature lymphoid cells are cultured in contact with stromal layers. Cells were cultured either in direct contact with stromal layers or separated from stroma by a microporous membrane (Transwell; Costar Corp.). Bars (mean ± SD of quadruplicate tests) represent percentage of cell recovery after 4 d with anti-CD38 antibody (T16) relative to parallel cultures without anti-CD38. Contact with stroma enhances growth suppression by anti-CD38 but is not a requisite.

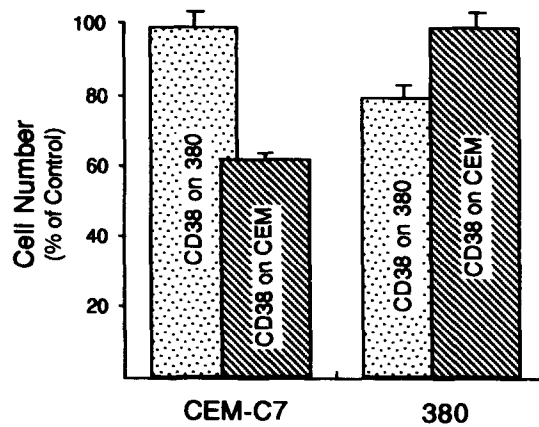


Figure 3. Suppressive effects of anti-CD38 are directly caused by antibody binding to immature lymphoid cells. CEM-C7 and 380 cells were labeled with anti-CD38 (T16) antibody in separate tubes. Excess antibody was removed and labeled cells of one line were mixed in equal numbers with unlabeled cells of the other line. Cell mixtures were seeded onto stromal layers for 48 h, and cell numbers were counted by flow cytometry after staining with CD7 (positive on CEM-C7) and CD19 (positive on 380). Bars represent percentage (mean ± SD of quadruplicate tests) of cell recovery relative to the number of cells in cultures without anti-CD38. Suppressive effects are seen only in cells labeled with anti-CD38 antibody.

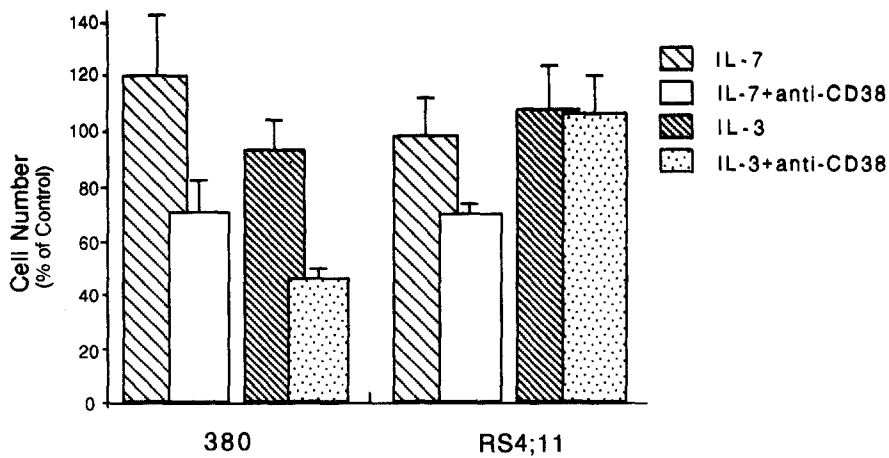


Figure 4. Induction of sensitivity of anti-CD38 in immature lymphoid cells by cytokines in the absence of stroma. Bars (mean \pm SD of the quadruplicate tests) represent percentage of cell recovery after 4 d of culture relative to parallel cultures without cytokines. IL-7 (25 ng/ml) had weak stimulatory effect on 380 cells, which, in turn, became sensitive to anti-CD38 (T16). Anti-CD38 antibodies also inhibited the growth of RS4;11 in the presence of IL-7. IL-3 (10 ng/ml) rendered 380 cells, but not RS4;11 cells, sensitive to anti-CD38.

without anti-CD38 (T16). Addition of anti-CD38 to cultures with IL-7 reduced cell recovery in all six cell lines, with a greatest effect in the 380 cells (Fig. 4). Recovery of 380 cells also decreased by >50% when anti-CD38 was added to cultures with IL-3, by >20% when SCF was the stimulant, and by >60% when all three cytokines were used together. IL-3 and SCF did not render cells sensitive to the suppressive effects of CD38 in the other five cell lines. Thus, the stromal factors that are essential to reveal the inhibitory effects of anti-CD38 on the growth of immature lymphoid cells can be partially replaced by stroma-derived cytokines.

Effects of CD38 Ligation on Cell Proliferation and Apoptosis. We assessed whether the suppressive effects of anti-CD38 antibodies on lymphoid progenitor cells were due to inhibition of cell proliferation and/or induction of programmed cell death (apoptosis). When the immature B cell line OP-1 was cultured on stromal layers in the presence of an anti-CD38 antibody (T16), there was a clear suppression of cell growth (Fig. 5 A). Suppression of cell proliferation was confirmed by DNA content analysis, which showed an \sim 50% decrease in the percentage of cells in the proliferative phases of the cell cycle, with a corresponding increase of cells in G0/G1, after culture with anti-CD38; similar observations were made with the cell line RS4;11 (Table 2).

The kinetics of anti-CD38-mediated effects were different in leukemic lymphoblasts from a patient with ALL. Addition of CD38 antibodies to stroma-supported cultures induced a dramatic reduction in cell number during the first 24 h of culture (Fig. 5 B). Cell numbers progressively declined during further culture. This suggested that CD38 ligation could exert a cytotoxic effect on progenitor cells in addition to the cytostatic effect seen with the cell lines. Thus, we quantitated the percentage of cells with fragmented DNA, a characteristic of apoptosis, after anti-CD38 addition to the cultures. In the case of ALL illustrated in Fig. 5 B, 44% of leukemic lymphoblasts had fragmented DNA after 24 h of culture with anti-CD38 versus 8% in control cultures. DNA fragmentation was also detectable after CD38 ligation in stroma-supported cultures of cell lines. After 24–48 h of culture, 18% apoptotic cells with anti-CD38 versus 8% in con-

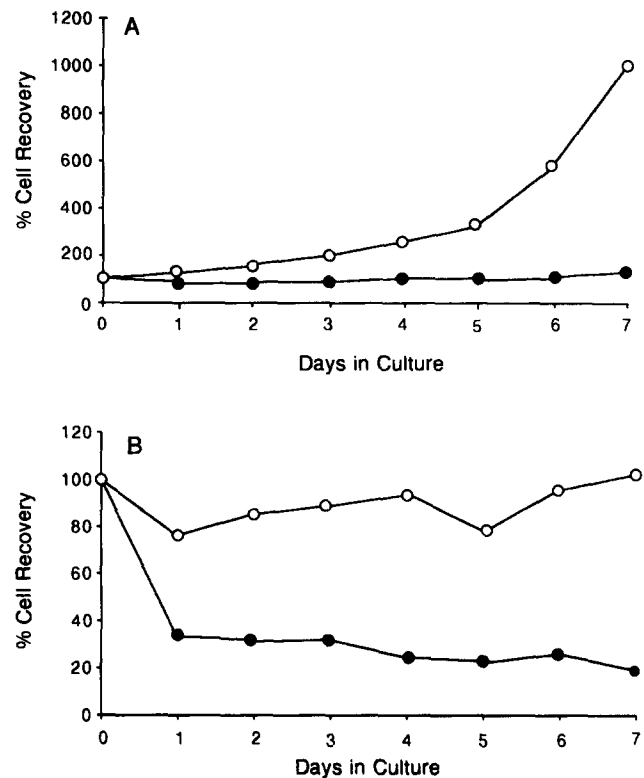


Figure 5. Kinetics of suppression of immature lymphoid cells by CD38 ligation. Cells from one immature B cell line (OP-1; A) and one case of ALL (B) were seeded onto stromal layers and harvested daily from separate wells, resuspended in equal volumes, and counted by flow cytometry. Results are expressed as percentage of viable CD19⁺ cells counted at different time points relative to those originally seeded. Empty circles indicate values (mean of triplicate counts; SD <10%) obtained in control cultures, while solid circles are values obtained in cultures containing anti-CD38 antibody (T16). In cultures of OP-1 (A), addition of anti-CD38 suppressed cell growth throughout the culture period. In leukemic lymphoblasts from a child with ALL (B), addition of CD38 antibody caused a rapid decline in cell numbers, which continued throughout the culture period.

Table 2. Distribution of Cells in Phases of the Cell Cycle after CD38 Ligation*

Phase	RS4;11		OP-1	
	Control	Anti-CD38	Control	Anti-CD38
	%		%	
G0/G1	75	89	57	77
S	22	10	40	22
G2/M	3	1	3	1

* DNA content analysis was performed after 48 h on stromal layers with anti-CD38 (T16) or isotype-matched control antibody. Three experiments were performed; results of one representative experiment are shown.

control cultures were seen with OP-1, while 29 versus 2% were detected with CEM-C7 (Fig. 6). These results show that CD38 ligation can suppress growth by both inhibition of DNA synthesis and induction of apoptosis.

Antibodies to CD38 Do Not Alter NAD⁺ Hydrolase or ADPR Cyclase Activity in Immature Lymphoid Cells. The amino acid sequence of CD38 is homologous to that of the enzyme ADP-ribosyl cyclase first isolated from the sea mollusk *Aphysia californica* (13), and CD38 catalyzes the conversion of NAD⁺ to nicotinamide and cADPR, and that of cADPR to ADPR (9, 15–19). It has been shown that cADPR is a costimulant of mitogenicity in murine lymphocytes (9). To investigate the effects of CD38 ligation on NAD⁺ hydrolase and cADPR production, we measured changes in extracellular levels of NAD⁺, nicotinamide, ADPR, and cADPR by HPLC. As illustrated in Fig. 7, NAD⁺ levels rapidly decreased when added to OP-1 cells. In parallel, nicotinamide, ADPR, and cADPR levels increased. In the presence of anti-CD38 antibodies, results were virtually identical to cells incubated without anti-CD38. Thus, neither NAD⁺ hydrolysis nor cADPR or ADPR production was affected by CD38 ligation.

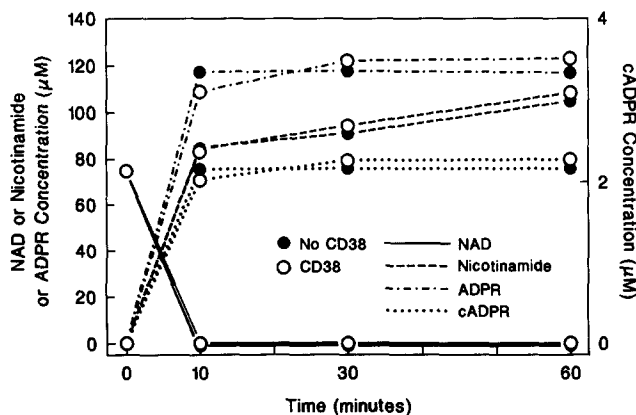


Figure 7. Assessment of NAD⁺ hydrolase and ADPR cyclase activity in OP-1 immature B cells in the presence or absence of anti-CD38 antibody. The disappearance of substrate (NAD⁺) and the appearance of products (nicotinamide, ADPR, and cADPR) was not affected by CD38-ligation.

tion. When no NAD⁺ was added to the cells, none of its metabolites was detectable.

The lack of effects on the enzymatic activity were corroborated by experiments in which the effects of nicotinamide, NAD⁺, ADPR, and cADPR on cell growth were tested. We added various concentrations of nicotinamide, NAD⁺, ADPR, and cADPR to stroma-supported cultures of normal bone marrow CD19⁺ cells (one sample), B lineage ALL cells (one sample), and the cell lines NALM6, OP-1 and RS4;11. Nontoxic concentrations of nicotinamide (5–500 µM), NAD⁺ (5–50 µM), ADPR (5–50 µM), and cADPR (2.5–100 µg/ml) neither changed cell recovery in the absence of CD38 antibodies nor offset the inhibitory effects of anti-CD38. The results of experiments with RS4;11 are shown in Fig. 8. These results suggest that the effects of CD38 ligation are not due to changes in the NAD⁺ hydrolase or ADPR cyclase activity of CD38.

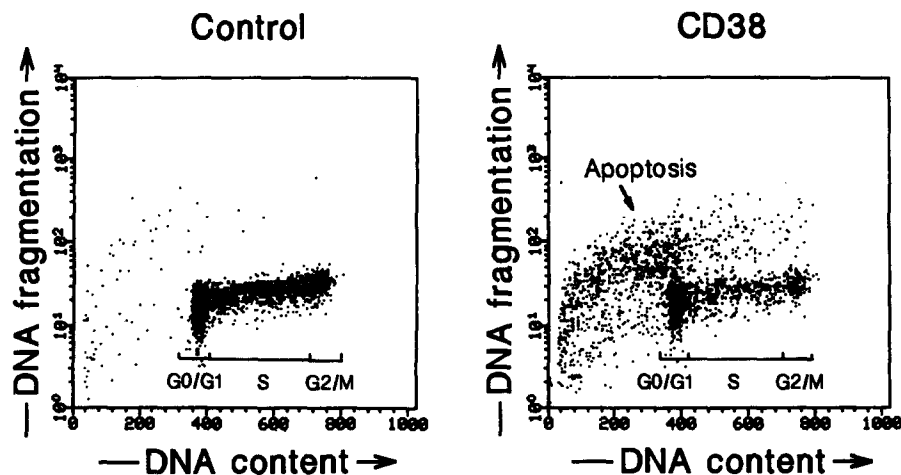


Figure 6. Ligation of CD38 induces apoptosis in immature lymphoid cells. Flow cytometric dot plots illustrate DNA content versus DNA fragmentation studied in CEM-C7 cells cultured for 24 h on stroma with or without anti-CD38 (T16). Anti-CD38 causes the appearance of cells with fragmented DNA and subnormal DNA content, characteristic of apoptosis.

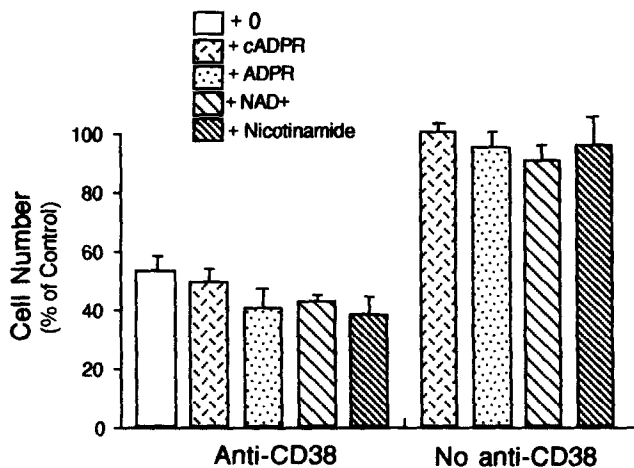


Figure 8. Effects of NAD⁺ (50 μ M), nicotinamide (500 μ M), ADPR (50 μ M), and cADPR (50 μ g/ml) on stroma-supported 2-d cultures of RS4;11 immature B cells in the presence or absence of anti-CD38 antibody (T16). Bars (mean \pm SD of quadruplicate tests) represent percentage of cell recovery relative to control cultures with no additives.

Discussion

This study demonstrated that ligation of CD38 inhibits the growth of human immature lymphoid cells. Antibodies to CD38 caused a drastic cell reduction in cultures of CD19⁺ B cells from normal bone marrow, leukemic cells from 22 ALL cases, and 8 immature lymphoid cell lines. The effects of anti-CD38 were apparent only under culture conditions approximating those of *in vivo* lymphopoiesis, that is, in the presence of factors derived from bone marrow stromal cells. In the absence of these microenvironmental stimuli, the inhibitory effects of anti-CD38 were not detectable.

The lack of inhibitory effects by anti-CD38 antibodies in the absence of stroma raised the possibility that the antibodies stimulated stromal elements to secrete factors that suppress lymphopoietic cell growth. However, only rare cells among stromal elements exhibited CD38 expression. In addition, CD38 ligation of cell lines before seeding on stroma was sufficient to inhibit cell growth, and no "bystander" effect could be seen in untreated cells added to the same cultures. Finally, growth was also partially inhibited by anti-CD38 when cells were exposed to cytokines in the absence of stroma. We conclude that the effects were due to a direct interaction between CD38 antibodies and lymphoid progenitors and were not indirectly caused by stimulation of stromal cells. The precise relationship between CD38 ligation and microenvironmental stimuli is still unknown. Sensitivity to the effects of anti-CD38 cannot be explained by increased intensity of CD38 expression because this did not substantially change when cells were cultured on stroma or in the presence of cytokines (data not shown). Although anti-CD38 antibodies also suppressed growth when cells were separated from stroma, the suppressive effect was greater when contact with stromal elements was allowed, suggesting that the stromal factors that render cells sensitive to anti-CD38 may be more active in

a membrane-bound form or present at more active concentrations in the extracellular matrix.

The suppressive effect of CD38 ligation on immature lymphoid cells contrasts markedly with the reported stimulatory and anti-apoptotic effects in human and murine mature lymphocytes (7–10). Another disparity of CD38 function in immature and mature lymphoid cells is its propensity to modulate its expression after incubation with anti-CD38 antibodies. In contrast to experiments with mature lymphocytes (12), we failed to detect capping or internalization of anti-CD38 in immature lymphoid cells even after 20 h of incubation (Kumagai M., and D. Campana, unpublished observations). These data taken together suggest that CD38 may have specific functions at different stages of cell maturation. Although CD38 does not display detectable biochemical differences among different cell types (34, 35), it has not been ruled out that different functional isoforms of CD38 exist and that they are expressed at different stages of maturation. This concept is supported by the observation of at least five different human CD38 transcripts (27) and two different transcripts for the murine homolog (36).

Our stroma-supported cultures of circulating and tonsillar B lymphocytes did not demonstrate the stimulatory effects of anti-CD38 antibodies reported by others. However, Santos-Argumedo et al. (8) observed that costimulation of a CD38-like antibody with IL-4 was necessary to reveal clear mitogenicity in resting murine B cells. Since no changes in cell numbers were seen when circulating B cells were challenged with anti-CD38 in our serum-free cultures, we conclude that bone marrow stroma itself does not act as a mitogenic costimulus of anti-CD38. We also failed to detect marked changes in numbers of tonsillar B cells labeled with anti-CD38. However, the short-term antiapoptotic effects on germinal center cells reported by Zupo et al. (10) with one of the antibodies used in our study (IB4) could have been masked by an antiapoptotic effect of the stromal cells themselves in our 7-d culture system.

The structural homology between CD38 and ADPR cyclase has raised the hypothesis that the function of CD38 on at least some cells could be related to production of cADPR, a compound that functions as a calcium-mobilizing agent (9). cADPR causes release of intracellular calcium in a variety of cells, including sea urchin eggs (37), pancreatic islet β cells (38), brain microsomes (39), and cardiac muscle (40). Although cADPR does not induce Ca²⁺ influx or surface antigen modulation, it appears to induce proliferation of murine lymphocytes stimulated with suboptimal concentrations of CD38, LPS, and IL-4 (9). Howard et al. (9) speculated that the physiologic role of CD38 in activated lymphoid cells could be the extracellular conversion of NAD⁺ into cADPR; cADPR would then be transported to an intracellular receptor or directly act through a yet-unidentified cell surface receptor. We tested whether a similar mechanism sustained the growth of lymphoid progenitor cells and whether anti-CD38 antibodies could alter CD38 enzymatic activity. However, the NAD⁺ hydrolase and ADPR cyclase activity of CD38-expressing cells did not appear to be affected by CD38 ligation.

tion. The alternative speculation that CD38 is internalized during lymphocyte activation and acts intracellularly does not seem plausible, since CD38 in progenitor cells is not internalized by antibody binding or by culture on stroma (data not shown). Above all, addition of cADPR to the cultures, even at the high concentrations necessary to stimulate murine lymphocytes (9), did not offset the suppressive effects of anti-CD38. These results suggest that, in lymphoid progenitors, CD38 could mediate signal transduction independently from its enzymatic activity.

In conclusion, these data indicate that CD38 may play a critical role in lymphoid cell growth in the bone marrow

microenvironment. Categories of molecules that are important in this setting include adhesion molecules, which participate in homing and stabilization of progenitor cells to bone marrow stromal cells, and growth factors and their receptors, which provide survival, proliferation, and differentiation signals (for reviews see references 41 and 42). CD38 interaction with a putative ligand would represent a new regulatory mechanism of B lymphopoiesis. We speculate that one physiologic role of CD38 ligation might be the conversion of a stimulatory signal, for example, a cytokine signal, into an inhibitory one. This would provide a rapid feedback mechanism for suppressing cytokine-stimulated lymphopoiesis.

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References

1. Reinherz, E.L., P.C. Kung, G. Goldstein, R.H. Levey, and S.F. Schlossman. 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc. Natl. Acad. Sci. USA.* 77: 1588-1592.
2. Terhorst, C., A. Van Agthoven, K. LeClair, P. Snow, E. Reinherz, and S. Schlossman. 1981. Biochemical studies of the human thymocyte cell-surface antigens T6, T9 and T10. *Cell.* 23:771-780.
3. Janossy, G., N. Tidman, S. Papageorgiou, P.C. Kung, and G. Goldstein. 1981. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. *J. Immunol.* 126:1608-1613.
4. Malavasi, F., F. Caligaris-Cappio, C. Milanese, P. Dellabona, P. Richiardi, and A.O. Carbonara. 1984. Characterization of a murine monoclonal antibody specific for human early lymphohemopoietic cells. *Hum. Immunol.* 9:9-20.
5. Sieff, C., D. Bicknell, G. Caine, J. Robinson, G. Lam, and M.F. Greaves. 1982. Changes in cell surface antigen expression during hemopoietic differentiation. *Blood.* 60:703-713.
6. Malavasi, F., A. Funaro, S. Roggero, A. Horenstein, L. Calosso, and K. Mehta. 1994. Human CD38: a glycoprotein in search of a function. *Immunol. Today.* 15:95-97.
7. Funaro, A., G.C. Spagnoli, C.M. Ausiello, M. Alessio, S. Roggero, D. Delia, M. Zaccolo, and F. Malavasi. 1990. Involvement of the multilineage CD38 molecule in a unique pathway of cell activation and proliferation. *J. Immunol.* 145:2390-2396.
8. Santos-Argumedo, L., C. Teixeira, G. Preece, P.A. Kirkham, and R.M.E. Parkhouse. 1993. A B lymphocyte surface molecule mediating activation and protection from apoptosis via calcium channels. *J. Immunol.* 151:3119-3130.
9. Howard, M., J.C. Grimaldi, J.F. Bazan, F.E. Lund, L. Santos-Argumedo, R.M.E. Parkhouse, T.F. Wälseth, and H.C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science (Wash. DC).* 262:1056-1059.
10. Zupo, S., E. Rugari, M. Dono, G. Taborelli, F. Malavasi, and M. Ferrarini. 1994. CD38 signalling by agonistic monoclonal antibody prevents apoptosis of human germinal center B cells. *Eur. J. Immunol.* 24:1218-1222.
11. Dianzani, U., A. Funaro, D. DiFranco, G. Garbarino, M. Bragardo, V. Redoglia, D. Buonfiglio, L.B. De Monte, A. Pileri, and F. Malavasi. 1994. Interaction between endothelium and CD4⁺CD45RA⁺ lymphocytes. Role of the human CD38 molecule. *J. Immunol.* 153:952-959.
12. Funaro, A., L.B. De Monte, U. Dianzani, M. Forni, and F. Malavasi. 1993. Human CD38 is associated to distinct molecules which mediate transmembrane signaling in different lineages. *Eur. J. Immunol.* 23:2407-2411.
13. States, D.J., T.F. Wälseth, and H.C. Lee. 1992. Similarities in amino acid sequences of Aplysia ADP-ribosyl cyclase and human lymphocyte antigen CD38. *Trends Biochem. Sci.* 17:495.
14. Galione, A. 1993. Cyclic ADP-ribose: a new way to control calcium. *Science (Wash. DC).* 259:325-326.
15. Kontani, K., H. Nishina, Y. Ohoka, K. Takahashi, and T. Katada. 1993. NAD glycohydrolase specifically induced by

- retinoic acid in human leukemic HL-60. *J. Biol. Chem.* 268:16895-16898.
16. Gelman, L., P. Deterre, H. Gouy, L. Boumsell, P. Debre, and G. Bismuth. 1993. The lymphocyte surface antigen CD38 acts as a nicotinamide adenine dinucleotide glycohydrolase in human T lymphocytes. *Eur. J. Immunol.* 23:3361-3364.
 17. Takasawa, S., A. Tohgo, N. Noguchi, T. Koguma, K. Nata, T. Sugimoto, H. Yonekura, and H. Okamoto. 1993. Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J. Biol. Chem.* 268:26052-26054.
 18. Summerhill, R.J., D.G. Jackson, and A. Galione. 1993. Human lymphocyte antigen CD38 catalyzes the production of cyclic ADP-ribose. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 335:231-233.
 19. Zocchi, E., L. Franco, L. Guida, U. Benatti, A. Bargellesi, F. Malavasi, H.C. Lee, and A. De Flora. 1993. A single protein immunologically identified as CD38 displays NAD⁺ glycohydrolase, ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities at the outer surface of human erythrocytes. *Biochem. Biophys. Res. Commun.* 196:1459-1465.
 20. Manabe, A., E. Coustan-Smith, F.G. Behm, S.C. Raimondi, and D. Campana. 1992. Bone marrow derived stromal cells prevent apoptotic cell death in B lineage acute lymphoblastic leukemia. *Blood.* 79:2370-2377.
 21. Wolf, M.L., J.A. Buckley, A. Goldfarb, C.L. Law, and T.W. LeBien. 1991. Development of a bone marrow culture for maintenance and growth of normal human B cell precursors. *J. Immunol.* 147:3324-3330.
 22. Ryan, D.H., B.L. Nuccie, and C.N. Abboud. 1992. Inhibition of human bone marrow lymphoid progenitor colonies by antibodies to VLA integrins. *J. Immunol.* 149:3759-3764.
 23. Manabe, A., K.G. Murti, E. Coustan-Smith, M. Kumagai, F.G. Behm, S.C. Raimondi, and D. Campana. 1994. Adhesion-dependent survival of normal and leukemic human B lymphoblasts on bone marrow stromal cells. *Blood.* 83:758-766.
 24. Manabe, A., E. Coustan-Smith, M. Kumagai, F.G. Behm, S.C. Raimondi, C.-H. Pui, and D. Campana. 1994. Interleukin-4 induces programmed cell death (apoptosis) in cases of high risk acute lymphoblastic leukemia. *Blood.* 83:1731-1737.
 25. Campana, D., A. Manabe, and W.E. Evans. 1993. Stroma-supported immunocytometric assay (SIA): a novel method for testing the sensitivity of acute lymphoblastic leukemia cells to cytotoxic drugs. *Leukemia.* 7:482-488.
 26. Dorken, B., P. Moller, A. Pezzutto, R. Schwartz-Albiez, and G. Moldenhauer. 1989. B-cell antigens: CD38. In *Leucocyte Typing IV*, W. Knapp, B. Dorken, W.R. Gilks, E.P. Rieber, R.E. Schmidt, H. Stein, and A.E.G.K. Von dem Borne, editors. Oxford University Press, Oxford, UK, 86.
 27. Stamenkovic, I., J. Stauton, and B. Seed. 1989. Molecular cloning of CD38. In *Leucocyte Typing IV*, W. Knapp, B. Dorken, W.R. Gilks, E.P. Rieber, R.E. Schmidt, H. Stein, and A.E.G.K. Von dem Borne, editors. Oxford University Press, Oxford, UK, 87.
 28. Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 630-631.
 29. Campana, D., E. Coustan-Smith, A. Manabe, M. Buschle, S.C. Raimondi, F.G. Behm, R. Ashmun, M. Arico, A. Biondi, and C.-H. Pui. 1993. Prolonged survival of B-lineage acute lymphoblastic leukemia cells is accompanied by overexpression of bcl-2 protein. *Blood.* 81:1025-1031.
 30. Gold, R., M. Schmied, G. Rothe, H. Zischler, H. Breitchopf, H. Wekerle, and H. Lassmann. 1993. Detection of DNA fragmentation in apoptosis: application of in situ nick translation to cell culture systems and tissue sections. *J. Histochem. Cytochem.* 41:1023-1030.
 31. Kumagai, M., A. Manabe, E. Coustan-Smith, R.L. Blakley, W.T. Beck, V.M. Santana, F.G. Behm, S.C. Raimondi, and D. Campana. 1994. Use of stroma-supported cultures of leukemic cells to assess antileukemic drugs. II. Potent cytotoxicity of 2-chloro-deoxyadenosine in acute lymphoblastic leukemia. *Leukemia.* 8:1116-1123.
 32. Lee, H.C., T.F. Walseth, G.T. Bratt, R.N. Hayes, and D.L. Clapper. 1989. Structural determination of a cyclic metabolite of NAD⁺ with intracellular CA²⁺-mobilizing activity. *J. Biol. Chem.* 264:1608-1615.
 33. MacLennan, I.C.M. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117-139.
 34. Alessio, M., S. Roggero, A. Funaro, L.B. De Monte, L. Peruzzi, M. Geuna, and F. Malavasi. 1990. CD38 molecule: structural and biochemical analysis on human T lymphocytes, thymocytes, and plasma cells. *J. Immunol.* 145:878-884.
 35. Jackson, D.G., and J.I. Bell. 1990. Isolation of a cDNA encoding the human CD38 (T10) molecule, a cell surface glycoprotein with an unusual discontinuous pattern of expression during lymphocyte differentiation. *J. Immunol.* 144:2811-2815.
 36. Harada, N., L. Santos-Argumedo, R. Chang, J.C. Grimaldi, F.E. Lund, C.I. Brannan, N.G. Copeland, N.A. Jenkins, A.W. Heath, R.M.E. Parkhouse, et al. 1993. Expression cloning of a cDNA encoding a novel murine B cell activation marker. *J. Immunol.* 151:3111-3118.
 37. Galione, A., H.C. Lee, and W.B. Busa. 1991. Ca²⁺-induced Ca²⁺ release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science (Wash. DC).* 253:1143-1146.
 38. Takasawa, S., K. Nata, H. Yonekura, and H. Okamoto. 1993. Cyclic ADP-ribose in insulin secretion from pancreatic β cells. *Science (Wash. DC).* 259:370-373.
 39. White, A.M., S.P. Watson, and A. Galione. 1993. Cyclic ADP-ribose-induced Ca²⁺ release from rat brain microsomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 318:259-263.
 40. Meszaros, L.G., J. Bak, and A. Chu. 1993. Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca²⁺ channel. *Nature (Lond.).* 364:76-79.
 41. Kincade, P.W., G. Lee, C.E. Pietrangeli, S.I. Hayashi, and J.M. Gimble. 1989. Cells and molecules that regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* 7:111-143.
 42. Rolink, A., and F. Melchers. 1991. Molecular and cellular origins of B lymphocyte diversity. *Cell.* 66:1081-1094.