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# A monoclonal antibody against a murine CD38 homologue delivers a signal to B cells for prolongation of survival and protection against apoptosis *in vitro*: unresponsiveness of X-linked immunodeficient B cells

Y. YAMASHITA,* K. MIYAKE,* Y. KIKUCHI,† K. TAKATSU,† S. NODA,‡ A. KOSUGI‡ & M. KIMOTO* **Department of Immunology, Saga Medical School, Nabeshima, Saga, †Department of Immunology, Institute of Medical Science, University of Tokyo, Tokyo, and ‡Biomedical Research Center, Osaka University Medical School, Osaka, Japan*

## SUMMARY

A novel monoclonal antibody (mAb) was established in an attempt to look for a cell-surface molecule that delivered a signal regulating apoptotic cell death of B cells. Because spleen cells in resting culture die from apoptosis, mAb were looked for that were able to prolong spleen cell survival *in vitro*. This screening selected mAb CS/2. CS/2 not only prolonged spleen cell survival *in vitro*, but also protected spleen cells from apoptotic cell death brought about by irradiation or dexamethasone. Moreover, stimulation of spleen cells with CS/2 mAb induced changes of cells to blastoid morphology, and a significant uptake of [$^3$H]thymidine ([$^3$H]TdR). The antigen recognized by CS/2 mAb (CS/2 Ag) was expressed on preB cells, B cells, and Mac-1$^+$ cells. The cells surviving *in vitro* culture or irradiation in response to ligation with CS/2 mAb were mostly B cells expressing the CS/2 Ag. In addition, B cells from X-linked immunodeficient (XID) mice did not respond to CS/2 mAb. These results indicate that CS/2 mAb is agonistic to B cells, and that XID mice are deficient in this CS/2 mAb-mediated activation pathway. Determination of the amino-terminal 24 amino acid residues revealed that the CS/2 Ag appears to be identical to the murine CD38 homologue. These results are consistent with the conclusion that CS/2 mAb is directed against a murine CD38 homologue, and suggest a possible role of the murine CD38 homologue in controlling apoptotic cell death of B cells.

## INTRODUCTION

B cells must survive several selection steps during their differentiation from immature to mature B cells or into antibody-secreting cells.[1,2] Only B cells that have experienced such selection are allowed to joint the mature B-cell pool[3–5] and, particularly in germinal centres, to differentiate into antibody-secreting cells.[6,7] These selection processes thus contribute to the maintenance of the stable B-cell pool without autoreactive B cells, and to efficient production of antibodies with adequate specificity and affinity against non-self antigens. In these selection steps, the induction of programmed cell death by apoptosis is likely to play a critical role, and the identification of a cell-surface molecule transducing a signal that controls apoptosis would profoundly facilitate the understanding of the selection system underlying B-cell maturation. We have attempted to establish monoclonal antibodies (mAb) against such molecules by a variety of screening strategies.

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Correspondence: Dr K. Miyake, Department of Immunology, Saga Medical School, Nabeshima, Saga 849, Japan.

In a previous study, we have described radioprotective mAb (RP mAb) that were able to protect B cells from irradiation-induced apoptosis.[8] These mAb were also able to protect B cells from dexamethasone-induced apoptosis, and to drive them into proliferation. B cells from X-linked immunodeficient (XID)[9] mice did not, however, respond to RP mAb. The antigen recognized by these mAb (RP105) was shown to be monomeric and to be expressed preferentially on mature B cells. RP105 would be a candidate for a signal transduction molecule that can control apoptotic cell death of B cells.

In this study, we attempted to look for mAb that were able to prolong spleen cell survival *in vitro*. Because spleen cells are reported to die from apoptosis when cultured *in vitro*,[10] prolongation of spleen cell survival by mAb would reflect the protective effect of mAb from apoptosis of spleen cells induced by *in vitro* culture. This screening strategy allowed us to select CS/2 mAb. The antigen recognized by the CS/2 mAb (the CS/2 Ag) was expressed mainly on B cells in spleen, and preB cells as well as B cells in bone marrow. In addition to prolongation of spleen cell survival, CS/2 mAb protected spleen cells from apoptosis induced by irradiation or dexamethasone as effectively as RP mAb. However, CS/2 mAb did not induce such a drastic proliferation of B cells as RP mAb did, but

induced moderate uptake of [$^3$H]thymidine ([$^3$H]TdR). B-cell activation resulting from CS/2 mAb was not observed with B cells from XID mice, as is the case with RP mAb.

The CS/2 Ag had a molecular weight of 45 000 MW, and determination of partial amino-terminal amino acid residues of CS/2 Ag showed complete identification to those of the murine CD38 homologue.[11] These results strongly suggest that CS/2 mAb is directed against the murine CD38 homologue. The signal through the murine CD38 homologue may be implicated in controlling apoptosis *in vivo* as well as *in vitro*.

## MATERIALS AND METHODS

*Cell lines and mice*

The B-cell lymphoma CH12[12] were a kind gift from Dr R. J. Hodes (National Institutes of Health, Bethesda, MD). The $BCL_1$ lymphoma cell[13] was from Dr E. Kubota (Department of Oral Surgery, Saga Medical School, Saga, Japan). The CH12 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 50 $\mu$M 2-mercaptoethanol (2-ME) and antibiotics. The $BCL_1$ cells were maintained in BALB/c mice *in vivo*. Mice were obtained from Japan SLC Co. (Hamamatsu, Japan) and maintained in the Animal Facility of Saga Medical School. Wistar rats were purchased from Charles River Japan Inc. (Tokyo, Japan). BALB/c.*xid* and C57BL/6.*xid* mice[14,15] were kindly provided by Dr A. Singer (National Institutes of Health) through Dr T. Hamaoka (Osaka University Medical School, Osaka, Japan) and maintained in the Animal Facility of the Institute of Medical Science (the University of Tokyo, Tokyo, Japan). All experiments were performed according to guidelines for the Care and Treatment of Animal Experimentation in the Saga Medical School and in the Institute of Medical Science.

*Monoclonal antibodies*

Wistar rats were immunized with spleen cells from BALB/c mice. Three days after the third immunization, spleen cells from an immunized rat were removed and fused with SP2/0 myeloma cells (American Type Culture Collection, Rockville, MD). The strategy for screening is described in the Results. The established mAb CS/2 is of the isotype IgG1/$\kappa$. The antibody against B220, RA3-6B2,[16] was obtained from Dr S. Murakami (Department of Periodontology, Osaka University Dental School, Osaka, Japan). The rat mAb against Mac-1 (M1/70.15.11.5.HL) was obtained from the American Type Culture Collection (ATCC). The rat antibodies against mouse IgM (AM/3; IgG2a $\kappa$), IgD (CS/15; IgG2a $\kappa$) and Thy-1 (GR/9; IgG2a $\kappa$) were established in this laboratory.[8] These antibodies were purified by ABx column chromatography (J. T. Baker Inc., Alexandria, VA) from ascites fluid prepared in CB17 *scid*/*scid* mice (Japan Clea Laboratories Inc., Tokyo, Japan).

*Cell survival assay, and protection from cell death by irradiation or dexamethasone*

For the cell survival assay, spleen cells from BALB/c or C57BL/6 mice ($2 \times 10^5$/well) were cultured in 96-well culture plates with or without the indicated antibodies (10 $\mu$g/ml), at 37° in humidified air with 5% $CO_2$. The culture medium consists of RPMI-1640 supplemented with 10% FCS, antibiotic and 50 $\mu$M 2-ME. Viable cells were determined by a trypan blue dye exclusion test. Percentages of viable cells were determined by the formula: percentage viable cells = 100 × (viable cell number/input cell number). The results are represented as mean ± SD for triplicate cultures.

For experiments of irradiation-induced cell death, spleen cells were placed in a 96-well plate ($2 \times 10^5$/well), and precultured with antibodies (10 $\mu$g/ml) for 2 hr. The cells were then irradiated with 100 or 200 rads by a $^{137}$Cs irradiator (Gamma cell 40; Atomic Energy of Canada Ltd, Radiochemical Co., Ontario, Canada) at a dose rate of 112 rads/min, and cultured for an additional 18 hr. As for dexamethasone-induced cell death, varying concentrations of dexamethasone (Wako Pure Chemical Industries Ltd, Osaka, Japan) were added to cultures simultaneously with antibodies, and cells were cultured for 20 hr. Viable cells were determined by a trypan blue dye exclusion test, and percentages of viable cells were determined as above. The results are represented as mean ± SD for triplicate cultures.

For measuring DNA content, cells were harvested and fixed in 70% ethanol. Fixed cells were stained with phosphate-buffered saline (PBS) containing 50 $\mu$g/ml propidium iodide (Sigma Chemical Co., St Louis, MO) and 10 $\mu$g/ml RNase (Nippon Gene, Toyama, Japan). After washing twice in PBS supplemented with 2% FCS and 0·1% sodium azide, cells were analysed on a FACScan® (Becton Dickinson Co., Mountain View, CA).

*Cell proliferation assay*

T-cell depleted spleen cells[14] were inoculated into a 96-well plate at $2 \times 10^5$/well, and cultured with 10 $\mu$g/ml mAb or lipopolysaccharide (LPS; *Escherichia coli* 055; B5; Difco Laboratories, Detroit, MI) for 3 days. The cultures were pulse-labelled with 1 $\mu$Ci [$^3$H]TdR (ICN Radiochemicals, Irvine, CA) for the last 6 hr of the culture. They were then harvested onto glass fibre filters and the incorporated radioactivity determined on a Betaplate flat-bed liquid scintillation counter (Pharmacia-Wallac, Gaithersberg, MD). The results are presented as mean c.p.m. ± SD from triplicate wells.

*Isolation of small B cells from spleen*

B cells were enriched from spleen by the panning technique. Plastic dishes were coated with 10 $\mu$g/ml mouse anti-rat $\kappa$ mAb (MAR18.5) in PBS at room temperature for 2 hr. After washing dishes with PBS, culture supernatant containing mAb against mouse CD4 and mouse CD8 was added to coated dishes. After washing out unbound mAb, spleen cells were added to the dishes and allowed to bind to coated mAb. The unbound population was collected. About 90% of the unbound population was positive for B220. From the unbound population, cells with $P > 1{\cdot}080$ were separated using Percoll (Pharmacia Biotech Inc., Piscataway, NJ), and used as small B cells for blast formation (Fig. 6).

*Immunofluorescence analysis*

Cells in suspension ($5 \times 10^5$ cells/200 $\mu$l) were incubated for 20 min on ice with mAb. After two washes, fluorescein isothiocyanate (FITC)-labelled mouse anti-rat $\kappa$ (MAR18.5; ATCC) was added for an additional incubation. Propidium iodide was included during this incubation period and used as a gating parameter to exclude dead cells. For dual staining, cells were incubated with a biotinylated antibody and, after washing twice, phycoerythrin (PE)-labelled avidin (Vector Laboratories

Inc., Burlingame, CA) and FITC-labelled antibody were added and incubated for 20 min on ice. In this case, propidium iodide was not included, but dead cells were excluded using the forward versus side scatter plot. Labelled cells were then analysed on a FACScan® (Becton Dickinson Co.).

*Cell surface biotinylation and immunoprecipitation*

Cell-surface biotinylation and immunoprecipitation were carried out as described previously.[17] Briefly, cells ($5 \times 10^7$/ml) were washed twice in Hanks' balanced salt solution (HBSS), and suspended in saline containing 100 mM HEPES (pH 8·0). Sulphosuccinimidobiotin (Pierce Chemical Co., Rockford, IL) was added to the cell suspension at a concentration of 0·5 mg/ml. After a 40-min incubation at room temperature with occasional shaking, cells were washed and lysed in a lysis buffer (see below). After a 30-min incubation on ice and following centrifugation, the cell lysate was recovered and incubated with mAb-coupled Sepharose 4B for 2 hr at 4°. After washing with lysis buffer, bound proteins were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto a nitrocellulose membrane, and visualized with avidin–peroxidase (Bio-Rad Laboratories, Richmond, CA) and an appropriate substrate.

*Micro-sequencing of amino-terminal amino acids of CS/2 Ag*

Purified CS/2 mAb (10 mg) was coupled to a normal human serum (NHS; N-hydroxysulfosuccinimide)-activated HiTrap column (1 ml) according to the manufacturer's instructions (Pharmacia P-L Biochemicals Inc., Milwaukee, WI). Cell lysate was prepared from $BCL_1$ cells ($3 \times 10^{10}$ cells/200 ml). Lysis buffer consisted of 50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 1% Triton X-100, 50 mM iodo-acetamide, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml soybean trypsin inhibitor (Wako Pure Chemical Industries Ltd), 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 0·1% sodium azide. The cell lysate was applied onto the CS/2 mAb-coupled column by overnight recirculation. The column was washed with buffer containing 20 mM Tris–HCl (pH 8·2), 0·1% Triton X-100, 150 mM NaCl, and 0·02% sodium azide. Bound proteins were eluted with buffer consisting of 50 mM glycine–HCl (pH 2·6) and 0·1% Triton X-100. Each fraction was immediately neutralized with 1 M Tris–HCl (pH 7·5). Peak fractions were combined and dialysed against 10 mM NaCl and 0·1% Triton X-100. After being lyophilized, purified protein was resolved by SDS–PAGE, electroblotted onto a polyvinyl difluoride (PVDF) membrane (Bio-Rad Laboratories), and stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories). The band of CS/2 Ag was excised and loaded to a protein sequencer (Applied Biosystems Inc., Foster City, CA). Homology was searched with the database of the National Center for Biotechnology Information (NCBI; Bethesda, MD) using a blast algorithm.[18]

## RESULTS

### A newly established mAb prolongs B-cell survival *in vitro*

In an attempt to select mAb that were able to prolong spleen cell survival *in vitro*, the following screening strategy was employed. Rats were immunized with spleen cells from BALB/c mice and rat spleen cells were fused with SP2/0 myeloma cells. Because the length of the cell survival assay made it impractical to use for the first screening of the mAb generated, a method employing differential staining was utilized instead. Normal spleen cells and a B-cell lymphoma, CH12, were stained with hybridoma supernatants, and those that stained normal spleen cells more brightly than CH12 were selected. This was based on the assumption that tumour cells might be expected to express less of a signal transduction molecule for survival than normal spleen cells, because tumour cells would not require the survival signal. The first screening selected five hybridomas, and these supernatants were then added to spleen cell cultures to examine their effect on spleen cell survival *in vitro*. One of them, CS/2 mAb, significantly prolonged spleen cell survival *in vitro*, and was, therefore, chosen for further study.

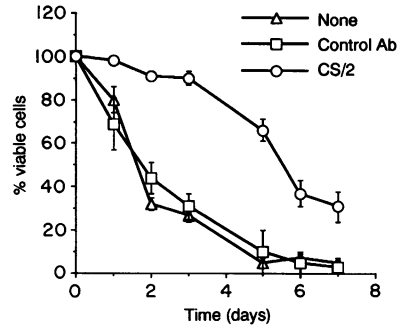


**Figure 1.** The effect of CS/2 mAb on spleen cell survival *in vitro*. Spleen cells were cultured with a control mAb (RA3-6B2), CS/2 mAb or without an antibody, as indicated in the figure, and viable cells were counted by a trypan blue dye exclusion. The antibodies were utilized at a concentration of 10 μg/ml. The results are presented as the mean percentages of viable cells ± SD for triplicate cultures.

Spleen cells from BALB/c mice were cultured with or without purified CS/2 mAb (10 μg/ml) and viable cells were counted everyday. As shown in Fig. 1, more than 70% of spleen cells died within 2 days when cultured alone or with an isotype-matched control mAb (rat anti-mouse CD45; RA3-6B2), whereas in the presence of the CS/2 mAb less than 20% of spleen cells died after 2 days and more than 30% of cells survived even on the seventh day of culture. These results

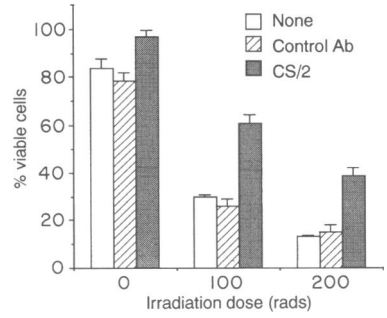


**Figure 2.** The radioprotective effect of CS/2 mAb. Spleen cells were precultured with the indicated antibodies (10 μg/ml) or without an antibody for 2 hr, and then irradiated at the doses indicated. After an additional 18-hr incubation, viable cells were counted by a trypan blue dye exclusion test. The results are presented as the mean percentages of viable cells ± SD for triplicate cultures. The control mAb was RA3-6B2.

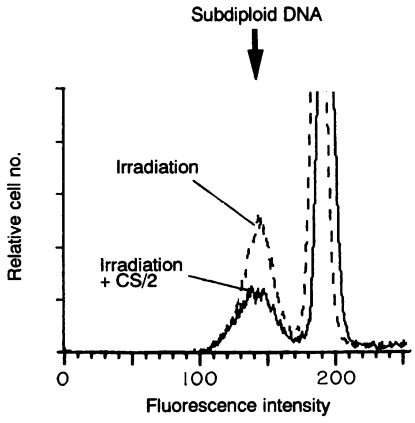


**Figure 3.** CS/2 mAb-reduced cells with subdiploid DNA represented as the percentage that were undergoing apoptosis upon irradiation. Spleen cells were incubated at 37° with or without CS/2 mAb (10 μg/ml) for 2 hr, and then irradiated at 200 rads. Cells were cultured again for an additional 6 hr and DNA contents were analysed as described in the Materials and Methods. Thirty thousand cells were analysed for each sample. Cells with subdiploid DNA are indicated in the figure. Detailed values with more controls are shown in Table 1.

suggested that CS/2 mAb prolonged spleen cell survival *in vitro*.

### CS/2 mAb protects spleen cells from cell death induced by irradiation or dexamethasone

Next, we studied the effect of the CS/2 mAb on apoptosis induced by irradiation. Spleen cells were treated with CS/2 mAb, then irradiated and cultured as described in the Materials and Methods. Viable cells were counted after a 20-hr culture. As shown in Fig. 2, incubation of the CS/2 mAb rescued cells from cell death brought about by irradiation. Since cells undergoing apoptosis can be identified by their DNA content,[19] the DNA content of irradiated cells cultured in the presence or

**Table 1.** Protection of spleen cells by CS/2 mAb from apoptosis by irradiation

| Spleen cell treatment | | | |
|---|---|---|---|
| CS/2 mAb | Irradiation (200 rads) | Cells with subdiploid DNA (%) | % subtracted |
| − | − | 7·4 | − |
| − | + | 28·3 | 20·9 |
| + | + | 17·1 | 9·7 |

Spleen cells were freshly prepared and incubated at 37° with or without CS/2 mAb (10 μg/ml). After the initial 2 hr, some cells were irradiated (200 rads) as indicated, and cultured again for an additional 8 hr. Cells were then harvested and stained with propidium iodide, as described in the Materials and Methods. DNA contents were analysed on a FACScan and the percentage of cells with subdiploid DNA (Fig. 4) is shown. Thirty thousand cells were analysed in each group. The percentage of cells with subdiploid DNA was 0·6% when cells were incubated at 4°.

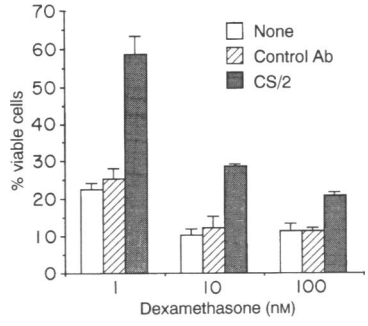


**Figure 4.** The protective effect from cell death of CS/2 mAb on spleen cells caused by dexamethasone. Spleen cells were cultured with varying amounts of dexamethasone, as indicated. Antibodies were present in some cultures at a concentration of 10 μg/ml. After 20 hr incubation, viable cells were counted by trypan blue dye exclusion. The results are presented as the mean percentages of viable cells ± SD for triplicate cultures. The percentage of viable cells in culture without dexamethasone or antibodies was 98·3 ± 4·5%. The control mAb was RA3-6B2.

absence of CS/2 mAb was examined in an attempt to study whether CS/2 mAb protected spleen cells from irradiation-induced apoptosis. As expected, culture with CS/2 mAb led to a reduction in the percentage of cells with subdiploid DNA (Fig. 3 and Table 1). Similarly, induction of cell death brought about by dexamethasone was blocked by CS/2 mAb (Fig. 4). Therefore, the effect of CS/2 mAb does not appear to be limited to spleen cell survival *in vitro*.

### CS/2 Ag is mainly expressed on B-lineage cells

The tissue distribution of the antigen recognized by CS/2 mAb (CS/2 Ag) was investigated. Cells from bone marrow, thymus, spleen and lymph nodes were stained with CS/2 mAb (data not shown). We could not find any positive cells in thymus. Positive cells were observed in bone marrow, spleen and lymph nodes. To examine this further, dual staining was carried out with spleen and bone marrow cells (Fig. 5). In bone marrow, 35% of the total nucleated cells were stained positive for both B220 and CS/2 Ag (Fig. 5a); 6·7% of bone marrow cells expressed B220 but not CS/2 Ag (Fig. 5a). The expression of B220 on this population appeared to be dull compared with cells positive for both B220 and CS/2 Ag. All IgM-positive cells (13·3%) expressed CS/2 Ag (Fig. 5c). A small population of Mac-1-positive cells (4·3% of total bone marrow cells) may have been dull-positive for CS/2 Ag (Fig. 5e). Collectively, in bone marrow, CS/2 Ag was mainly expressed on B-lineage cells, including some preB cells and surface IgM-positive B cells. Staining with other early B-cell markers will be required to determine the exact maturation stage when B-lineage cells acquire CS/2 Ag. In the spleen, CS/2 Ag-positive cells appeared to be divided into dull and bright populations, with CS/2 $Ag^{dull}$ and CS/2 $Ag^{bright}$ populations representing 17% and 43% of the total spleen cells, respectively. B220-positive and surface IgM-positive B cells appeared to belong to the bright population (Fig. 5b,d). Mac-1-positive cells were contained in both the dull and bright populations, at levels of 4% and 3%, respectively (Fig. 5f). Dual staining with CS/2 mAb and T-cell markers was carried out, and this indicated that T cells did not

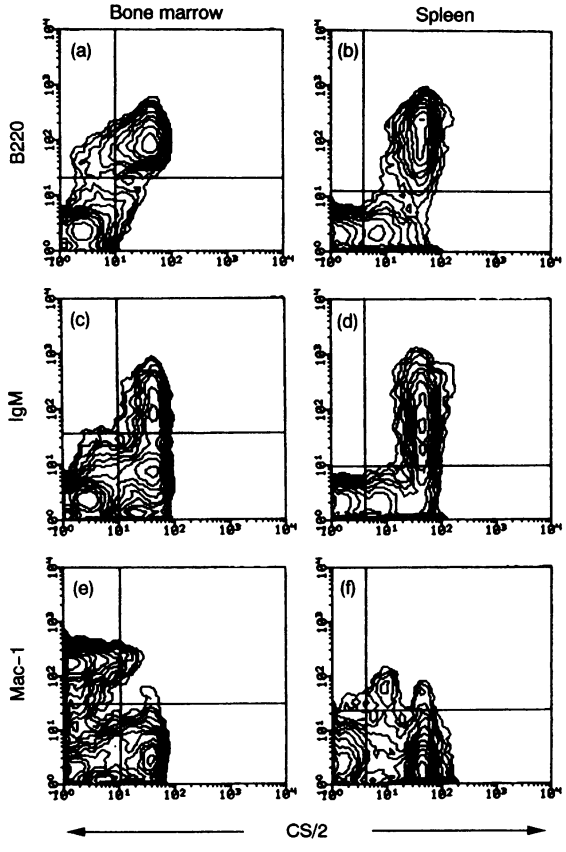


**Figure 5.** Dual staining of cells from bone marrow and spleen with CS/2 mAb and cell-surface markers. Cells from bone marrow (a, c, e) or spleen (b, d, f) were stained with FITC-labelled CS/2 antibody and biotinylated antibodies against B220 (a, b), IgM (c, d) or Mac-1 (e, f). Biotinylated antibodies were visualized with PE-labelled avidin. Forward and side scatters were used as gating parameters to examine the total nucleated cells for bone marrow cells, and lymphoid cells for spleen cells. Thirty thousand cells were analysed.

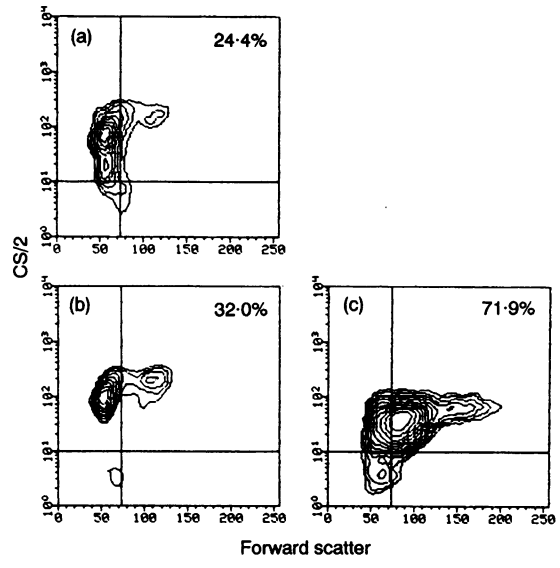


**Figure 6.** A blastic change of spleen cells induced with CS/2 mAb. T-cell depleted small B cells were prepared as in the Materials and Methods. Small B cells were cultured alone (b) or with CS/2 mAb (c) for 3 days, harvested, and then stained with CS/2 mAb followed by the second reagent. The horizontal axis shows the forward scatter reflecting the cell size, and the vertical axis corresponds to the fluorescence intensity (log) of CS/2 Ag. The profile of the starting population is shown in (a). Percentages of CS/2 Ag-positive blast cells (upper right regions) are shown. Thirty thousand cells were analysed for each sample.

seem to express CS/2 Ag (data not shown). Thus, as in bone marrow, the CS/2 Ag appeared to be mainly expressed on B cells in the spleen.

### CS/2 mAb induces blastic change and moderate uptake of [$^3$H]TdR in responding cells

To determine what kind of cells were responding to CS/2 mAb, we first examined whether CS/2 mAb could induce proliferation. We observed that spleen cells cultured with CS/2 mAb turned to blast cells, and that these blast cells could be taken to those cells that had responded to CS/2 mAb. We took the advantage of the fact that blast cells can be easily detected on a forward scatter plot by flow cytometry. T-cell-depleted small B cells were cultured with or without CS/2 mAb for 3 days, and the expression of the CS/2 Ag was depicted against forward scatter (Fig. 6). Two to three times more cells were recovered from culture with CS/2 mAb (data not shown). Moreover, blast cells in culture with CS/2 mAb increased in percentage compared with a resting culture without mAb. After culture with CS/2 mAb, all blast cells appeared to be positive for CS/2 Ag (Fig. 6c). These results indicated that the newly generated blast cells appeared to be CS/2 Ag-positive cells. Thus, CS/2 mAb is agonistic, and delivers a signal to CS/2 Ag-positive cells probably through CS/2 Ag. Blast cells were then stained with mAb against B-cell markers, including B220, IgM and IgD. Most blast cells expressed these B-cell markers (data not shown). Cells surviving *in vitro* culture or irradiation were also characterized by immunofluorescence analysis. Cells expressing B220, IgM and IgD increased in percentage, while Thy-1-positive cells decreased (data not shown). These results strongly suggest that CS/2 mAb is agonistic to B cells.

Spleen cells (Table 2) or T-cell-depleted spleen cells (Table 3) were cultured with CS/2 mAb, and [$^3$H]TdR uptake examined. These cells showed moderate but significant uptake of [$^3$H]TdR by stimulation with CS/2 mAb (Tables 2 and 3). The uptake of [$^3$H]TdR peaked around days 2–4 (data not shown). A control antibody did not induce any incorporation of [$^3$H]TdR (Table 3). We also used small B cells separated by panning and Percoll gradient sedimentation as responding cells, and had similar results with spleen cells and T-cell-depleted spleen cells (data not shown). Unexpectedly, cross-linking of CS/2 mAb with the second antibody (MAR18.5) or immobilization of CS/2 mAb on the plastic surface reduced the B-cell activation by CS/2 mAb (Table 2). It should be noted that MAR18.5 mAb is the mAb used for staining as the second reagent; therefore MAR18.5 mAb did not affect the interaction between CS/2 mAb and spleen cells. Interleukin-4 (IL-4) was added together with CS/2 mAb because IL-4 was shown to augment the response of B cells by the stimulation with CD38.[11,20] About a twofold increase in uptake of [$^3$H]TdR was

**Table 2.** The response of spleen cells to CS/2 mAb and its reduction by cross-linking or immobilization of CS/2 mAb

| | [$^3$H]TdR uptake (c.p.m.) | |
|---|---|---|
| mAb (10 $\mu$g/ml) | Exp. 1 | Exp. 2 |
| None | 661 ± 82 | 288 ± 34 |
| CS/2 (soluble) | 4631 ± 225 | 6448 ± 78 |
| CS/2 (immobilized) | 531 ± 96 | ND |
| MAR18.5 mAb | 560 ± 209 | 349 ± 89 |
| CS/2 + MAR18.5 | 600 ± 186 | 389 ± 85 |

Spleen cells were inoculated into a 96-well microtitre plate ($2 \times 10^5$ cells/well) and cultured for 3 days with the indicated mAb. For immobilization, purified CS/2 mAb (10 $\mu$g/ml) in PBS was added to the wells and incubated for 2–3 hr at room temperature. After washing unbound mAb with HBSS, spleen cells were added to coated wells. For cross-linking, MAR185. mAb (mouse anti-rat $\kappa$) was added simultaneously. Cells were pulsed 1 $\mu$Ci [$^3$H]TdR for the last 6 hr of the culture, and cell-associated c.p.m. were measured. The values are the mean ± SD for triplicate cultures.

ND, not determined.

**Table 3.** B cells from XID mice do not respond to CS/2 mAb

| | [$^3$H]TdR uptake stimulated with (10 $\mu$g/ml) | | | |
|---|---|---|---|---|
| Mice | None | Control Ab | CS/2 | LPS |
| BALB/c | 103 ± 16 | 132 ± 35 | 3354 ± 48 | 33 067 ± 3167 |
| BALB/c.*xid* | 58 ± 15 | 108 ± 28 | 49 ± 18 | 3 364 ± 375 |
| C57BL/6 | 89 ± 2 | 137 ± 22 | 3331 ± 308 | 24 172 ± 2008 |
| C57BL/6.*xid* | 64 ± 23 | 44 ± 24 | 47 ± 26 | 32 533 ± 246 |

T-cell depleted spleen cells were inoculated into a 96-well microtitre plate and cultured with antibodies or LPS for 3 days. Anti-CD45 (RA3-6B2) mAb was used as a control antibody. Cells were pulsed with 1 $\mu$Ci [$^3$H]TdR for the last 6 hr of culture, and cell-associated c.p.m. were measured. The values are the mean ± SD for triplicate cultures.

observed (data not shown). Concerning antibody secretion, no significant production of polyclonal IgM was observed by CS/2 mAb, as judged by enzyme-linked immunosorbent assay (ELISA) (data not shown).

### B cells from X-linked immunodeficient mice do not respond to CS/2 mAb

We then examined B cells from XID mice, because these B cells show a defective response to stimulation with RP mAb.[8] B cells from wild-type mice showed a moderate but significant response to CS/2 mAb, but those from XID mice did not respond (Table 3). Varying amounts of CS/2 mAb were tested for activation of wild-type or XID B cells (Table 4). Wild-type B cells responded well to as little as 0·5 $\mu$g/ml CS/2 mAb, while XID B cells did not respond to even as much as 5 $\mu$g/ml or

**Table 4.** Dose–response of wild-type and XID B cells to CS/2 mAb

| | [$^3$H]TdR uptake (c.p.m.) | |
|---|---|---|
| | Responding cells | |
| CS/2 mAb ($\mu$g/ml) | C57BL/6 | C57BL/6.*xid* |
| 0 | 60 ± 2 | 56 ± 32 |
| 0·5 | 1678 ± 125 | 91 ± 7 |
| 5·0 | 1555 ± 392 | 90 ± 7 |

T-cell-depleted spleen cells were cultured ($1 \times 10^5$/well) with or without CS/2 mAb for 3 days. The cultures were pulsed with 0·2 $\mu$Ci/well [$^3$H]TdR for the last 6 hr of the culture. The values are the mean ± SD for triplicate cultures.

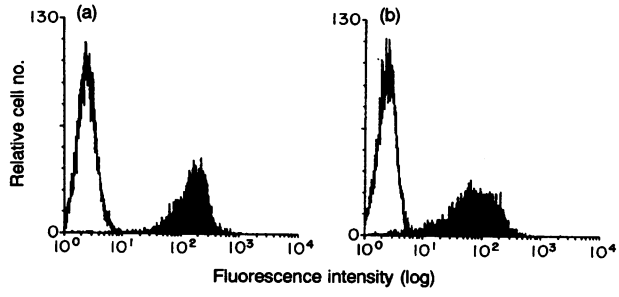


**Figure 7.** Expression of the CS/2 antigen on B220-positive B cells with XID. Spleen cells were stained with CS/2 mAb and anti-B220 (RA3-6B2). B220-positive cells were gated and expression of the CS/2 antigen is shown (filled profiles). Open profiles are those stained with the second reagent alone. (a) C57BL/6 mice; (b) C57BL/6.*xid* mice.

10 $\mu$g/ml (Table 3) CS/2 mAb. Thus larger amounts of CS/2 mAb did not overcome the defective response of XID B cells. This lack of responsiveness could not be attributed to lack of expression of the CS/2 Ag, since B220-positive B cells from XID mice expressed the CS/2 antigen (Fig. 7).

### Determination of the amino acid sequence of CS/2 Ag, and identification as the murine CD38 homologue

To characterize the biochemical properties of the CS/2Ag, the apparent molecular weight of CS/2 Ag was determined by immunoprecipitation. A single band was detected in both reduced and non-reduced conditions, which was approximately 45 000 MW under reduced conditions (Fig. 8). Then we tried to identify CS/2 Ag by determining a partial amino-terminal amino acid sequence. CS/2 Ag was purified using CS/2 mAb-coupled beads, resolved on SDS–PAGE, and blotted onto a PVDF membrane. The corresponding band was excised, and loaded to a protein sequencer. As shown in Fig. 9, the amino-terminal 24 residues were determined. This sequence was confirmed by two additional microsequencing experiments. A homology search of the NCBI data base using the blast algorithm[18] showed significant homology of CS/2 Ag to the human CD38[21] and complete identity with the amino-terminal

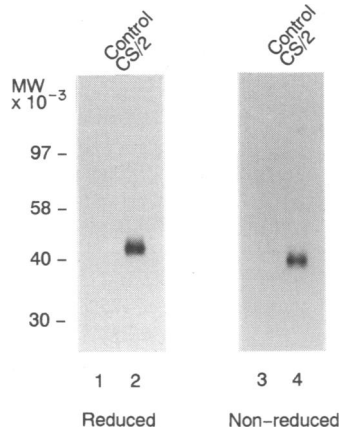


**Figure 8.** Immunoprecipitation of the CS/2 antigen. Cell-surface proteins on $BCL_1$ cells were biotinylated with sulpho–NHS–biotin, extracted with detergent, and immunoprecipitated with beads alone (lanes 1 and 3) or with CS/2 mAb-coupled beads (lanes 2 and 4). Precipitated proteins were subjected to SDS–PAGE (9% acrylamide), and visualized after blotting onto a nitrocellulose membrane.

| | 1 | 10 | 20 | 25 |
|---|---|---|---|---|
| CS/2 antigen: | ANYEFSQVS | GDRPGXRLSR | KAQIGL | |
| Mouse CD38 homologue: | MANYEFSQVS | GDRPGCRLSR | KAQIGL | |

**Figure 9.** Partial amino acid sequence of the CS/2 Ag and its identity with the murine CD38 homologue. The amino acid sequence of the $NH_2$-terminal of CS/2 Ag was determined as described in the Materials and Methods, and is shown in single letter code. The letter X denotes an undefined residue.

region of the murine CD38 homologue recently published by Harada *et al.*[11] The absence of an initiating methionine in CS/2 Ag compared with the murine CD38 homologue may be explained by a removal from a nascent chain during the processing of proteins.[22] In addition to the partial amino acid sequence, the expression pattern and functional properties of CS/2 Ag were basically compatible with those of the murine CD38 homologue.[20] Accordingly, the target molecule recognized by CS/2 mAb is most likely to be the murine CD38 homologue. Formal identification may require cDNA cloning and sequencing of CS/2 Ag.

## DISCUSSION

The new mAb CS/2 was shown to prolong B-cell survival *in vitro*, and to protect B cells from apoptosis induced by irradiation or dexamethasone. B-cell activation by CS/2 mAb resulted in blastgenesis and moderate uptake of [$^3$H]TdR. Most blast cells responding to CS/2 mAb expressed B220, IgM and IgD, as well as CS/2 Ag. Moreover, B cells from XID mice did not respond to CS/2 mAb. These results suggest that CS/2 mAb delivers a signal into B cells, resulting in activation and protection from apoptotic cell death. CS/2 Ag is 45 000 MW, and 24 residues of amino-terminal amino acids are identical with those of the murine CD38 homologue that has been recently identified by mAb NIM-R5[20] and subsequent cloning of a cDNA.[11] NIM-R5 mAb against murine CD38 homologue activated B lymphocytes, and rescued already activated B cells from apoptosis brought about by *in vitro* culture.[20] These results with NIM-R5 mAb appear to be consistent with those with CS/2 mAb in the present study, and strongly demonstrate that CS/2 mAb is directed against murine CD38 homologue.

The findings above suggest that murine CD38 homologue itself delivers a signal into B lymphocytes, and mAb against the murine CD38 homologue may imitate a physiological ligand. Recently, the murine CD38 homologue was reported to have an enzymatic activity catalysing the synthesis and hydrolysis of cyclic ADPribose (cADPR).[23] This is a metabolite of a nicotinamide adenine dinucleotide ($NAD^+$), and has calcium-mobilizing activity in sea urchin egg microsomes[24] as well as mammalian cells.[25,26] cADPR was demonstrated to have a synergistic effect with mAb against the murine CD38 homologue, although cADPR by itself had little effect on lymphocytes in terms of $Ca^{2+}$ influx.[23] B-cell activation with CS/2 mAb may be mediated by modulation of enzymatic activities of murine CD38 homologue by CS/2 mAb.

An alternative explanation for the agonistic activity of CS/2 mAb comes from the findings regarding human CD38. It has been reported that mAb against CD38 delivered an activation signal into thymocytes, T-cell lines, myeloma cells and natural killer cells.[27] Recently, association of human CD38 was shown with signalling molecules, including the CD3–T-cell receptor complex on T cells, surface immunoglobulin and the complement receptor 2 (CR2)–CD19 complex on B cells, and the low affinity IgG Fc receptor (FC$\gamma$RIII–CD16) on natural killer cells.[28] Funaro *et al.* proposed that human CD38 belonged to these signalling complexes.[28] These findings raise a possibility that the effect of CS/2 mAb is mediated by a signalling molecule that is associated with murine CD38 homologue.

Mice with XID lack a mature B-cell population, which normally develops late in ontogeny, and have defective responsiveness to B-cell stimuli through surface immunoglobulin receptors and cytokine responses.[9,14,29] B-cell maturation seems to be arrested. Recently, a gene involved in XID was identified.[30,31] The gene, *btk*, encodes a member of the *src* family of protein tyrosine kinases. A single conserved amino acid residue within the amino-terminal unique region of Bruton's tyrosine kinase (Btk) is mutated in XID mice.[32] Btk is supposed to work in signal transmission, which is indispensable for B-cell maturation. The result that B cells with XID defect do not respond to CS/2 mAb may suggest that the signal by CS/2 mAb requires Btk for transmission.

Dual staining with CS/2 mAb and cell-surface markers revealed that Mac-1-positive cells express CS/2 Ag. We have not yet examined whether CS/2 mAb deliver a signal into Mac-1-positive cells or not. In our system, almost all cells responding to CS/2 mAb would be B cells, because XID mice do not show any response to CS/2 mAb. However, as only 4–6% of spleen cells express both Mac-1 and CS/2 Ag, cell enrichment would be required for final confirmation.

In conclusion, we have established a mAb, CS/2, that prolongs B-cell survival *in vitro* and protects B cells from apoptosis induced by irradiation and dexamethasone. This antibody is directed against the murine CD38 homologue. These results suggest a role of the murine CD38 homologue in controlling apoptosis of B cells.

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