Promotion of IL-4- and IL-5-dependent differentiation of anti-μ-primed B cells by ascorbic acid 2-glucoside

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Abstract

The stable ascorbic acid derivative 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) was used to investigate the role of ascorbic acid (AA) in B cell differentiation *in vitro*. AA-2G is stable in a solution unlike AA but is hydrolyzed by cellular α-glucosidase to release AA. Mouse spleen B cells were primed for 2 days with an anti-μ antibody in the presence of interleukin (IL)-4 and IL-5 and then washed and recultured with AA-2G in the presence of IL-4 and IL-5. AA-2G, but not AA, dose-dependently increased IgM production, the greatest enhancement being 150% at concentrations of more than 0.5 mM. In the absence of IL-4 and IL-5, primed B cells produced a negligible amount of IgM, and AA-2G had no effect. AA-2G-induced IgM production in the presence of IL-4 and IL-5 was inhibited by the α-glucosidase inhibitor castanospermine. Intracellular AA content, depleted during the priming period, increased by adding AA-2G at the start of reculture. Treatment of B cells with AA-2G resulted in an increase in the number of IgM-secreting cells, CD138-positive cells and CD45R/B220-negative cells. The number of viable cells in untreated cultures decreased gradually, but the decrease was significantly attenuated by AA-2G, resulting in about 70% more viable cells in AA-2G-treated cultures. AA-2G caused a slight but reproducible enhancement of DNA synthesis and a slight decrease in the number of cells with a sub-G1 DNA content. These results demonstrated that AA released from AA-2G enhanced cytokine-dependent IgM production in anti-μ-primed B cells and suggest that its effect is caused through promoting the differentiation of B cells to plasma cells and attenuating the gradual decrease in the number of viable cells.

1. Introduction

Cells of the immune system produce a large amount of reactive oxygen species as part of their normal functions such as chemotactic locomotion and phagocytosis. Whereas reactive oxygen species play an essential role in the intracellular killing of bacteria and other invading organisms, immune cells are sensitive to reactive oxygen species because of the high percentage of polyunsaturated fatty acids in plasma membranes of those cells [1]. A high cellular concentration of the antioxidant ascorbic acid (AA) in immune cells might contribute to the prevention of cellular damage mediated by reactive oxygen species and might have a modulatory role in the process of immune response [2]. In fact, several studies have shown *in vitro* and *in vivo* immunostimulatory effects of AA. Supplementation with AA in healthy subjects enhanced neutrophil chemotaxis and T lymphocyte proliferation [3-5]. Administration of AA in patients exposed to toxic chemicals enhanced natural killer (NK) cell activity and improved lymphocyte blastogenic responses to T and B cell mitogens [5-8]. *In vitro* treatment with AA was shown to stimulate phagocytosis of peritoneal macrophages and NK cell activity [9]. However, there have been few studies on the effects of AA on antibody production, and it is unknown whether or not B cell differentiation is promoted by AA. *In vitro* differentiation of B cells into antibody-producing plasma cells requires a culture period of several days, during which time exogenously added AA in a medium is oxidatively degraded and intracellular AA is depleted. Thus, the instability of AA in an aqueous solution is a major problem to use it in experiments of *in vitro* antibody production.

We have synthesized an AA derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic

acid (AA-2G), that is stable in a solution unlike AA but is hydrolyzed by cellular α-glucosidase to release AA [10-13]. In order to determine the role of AA in B cell differentiation, we investigated in this study whether AA-2G augments cytokine-dependent antibody production in *in vitro* anti-μ-primed B cells enriched from murine splenocytes. The results showed that AA released from AA-2G potently enhanced production of IgM by anti-μ-primed B cells through promoting the differentiation of B cells to plasma cells and attenuating a decrease in the number of viable cells.

2. Materials and methods

2.1. Reagents

AA-2G was donated from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). AA-2G was dissolved in phosphate-buffered saline, neutralized with NaOH, adjusted to 100 mM, and filtered before use. The $F(ab')_2$ fragment of goat anti-mouse IgM antibody (Ab) (μ chain-specific) was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Recombinant mouse interleukin (IL)-4 and IL-5 were purchased from R&D Systems (Minneapolis, MN, USA). L-Ascorbic acid 2-phosphate (AA-2P) sesquimagnesium salt, L-ascorbic acid 2-sulfate (AA-2S) dipotassium salt, and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Castanospermine and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were pruchased from Wako Pure Chemical Industries (Osaka, Japan) and Calbiochem (La Jolla, CA, USA), respectively. Purified mouse IgM, goat anti-mouse IgM Ab, and horseradish peroxidase (HRP)-conjugated goat

anti-mouse IgM Ab were obtained from Zymed Laboratories Inc (San Francisco, CA, USA), Organon Teknika Corporation (Durham, NC, USA), and Kirkegaad & Perry Laboratories (Gaithersburg, MD, USA), respectively. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45R/B220, phycoerythrin (PE)-conjugated rat anti-mouse CD138 (syndecan-1), and anti-mouse CD16/CD32 monoclonal Abs (mAbs) were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). [6-^{3}H]Thymidine was obtained from GE Healthcare Bio-Sciences Corp. (Little Chalfont, UK).

2.2. Mice

Female BALB/c mice, purchased from Charles River Japan (Yokohama, Japan), were maintained under specific pathogen-free conditions in the animal facility of Okayama University and used between 8 and 12 weeks of age. Experimental procedures involving mice were approved by the Animal Research Control Committee of Okayama University.

2.3. Preparation of enriched B cells

Murine spleen resting conventional ($CD5^{-}$) B cells were enriched by negative selection using a mouse B lymphocyte enrichment set (BD Biosciences Pharmingen) as decribed previously [14]. Briefly, murine spleen cells depleted of erythrocytes by lysis of erythrocytes with ammonium chloride were suspended in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated on plastic dishes for 90 min at 37 °C. The nonadherent cells were then collected, suspended in the

same medium, and incubated on ice for 60 min with biotin-conjugated anti-mouse CD4, CD43 and TER-119/erythroid cell mAbs. The cells bearing the biotinylated antibodies were bound to BD IMag streptavidin particles, and negative selection was then performed on a BD IMagnet according to the manufacturer's protocol to enrich unlabeled B cells. The purity of recovered viable B cells ranged between 97% and 98% when the cells were stained with FITC-conjugated anti-mouse CD45R/B220 mAb and PI after preincubation of the cells with anti-mouse CD16/CD32 mAb to block Fc-mediated binding of Ab to the Fcγ receptor of cells and analyzed by a flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA). More than 95% cells in the enriched B cell preparations were small, resting B cells as judged by the cell size (forward scatter) in flow cytometry.

2.4. Antibody response of enriched B cells stimulated with anti-μ, IL-4 and IL-5

A two-step culture system described previously was used [15,16]. Enriched B cells were suspended in basal culture medium (RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin G and 100 μg/mL of streptomycin) and primed with anti-μ (10 μg/mL) in the presence of IL-4 (5.6 ng/mL) and IL-5 (6 ng/mL) for 2 days in flat-bottom 96-well plates (1.2×10^6 cells/200 μL/well) at 37 °C in an atmosphere containing 5% CO_2. The cells were washed twice with the basal culture medium to remove anti-μ and then recultured with or without AA or AA-2G in the presence of IL-4 (5.6 ng/mL) and IL-5 (6 ng/mL) for the indicated days in triplicate in round-bottom 96-well plates at a density of 1×10^5 cells/200 μL/well. The culture supernatant was then collected and was frozen at −30 °C for an IgM ELISA. The IgM levels were assayed by a sandwich ELISA essentially as

described previously [17] .

2.5. Enzyme-linked immunospot (ELISPOT) assay for detection of polyclonal IgM-secreting cells

The ELISPOT assay was performed as described previously [18] . Briefly, each well of 96-well microtiter plates was coated with 50 μL of goat anti-mouse IgM Ab (10 μg/mL) by being incubated overnight at 4 °C and then washed three times with PBS containing 0.05% Tween-20. The plates were blocked with 200 μL of 1% BSA in PBS for 2 h at room temperature and washed. To those plates, enriched B cells, which had been incubated with or without AA-2G for 4.5 days in the reculture period and washed with and suspended in the basal culture medium, were added (1×10^3 viable cells/200 μL/well) in triplicate and incubated for 12 h at 37 °C. After being washed three times, the plates was incubated with 50 μL of HRP-conjugated goat anti-mouse IgM Ab (0.2 μg/mL) for 1 h at room temperature. After washing, 100 μL of substrate solution consisting of 50 mM sodium phosphate buffer (pH 7.0), 1% agarose, 2 mg/mL of diaminobenzidine, 0.0018% H_2O_2, 0.0018% $NiCl_2$, and 0.0018% $CoCl_2$ was added. The plates were then incubated overnight at 37 °C in the dark, and the number of spot-forming cells was counted under a stereomicroscope. The data are expressed as number of spot-forming cells per culture.

2.6. Flow cytometric analysis of cell surface antigen expression

Enriched B cells, which had been incubated with or without AA-2G for 4 days in the reculture period, were pooled and washed with and suspended in PBS

containing 0.5% BSA and 0.1% sodium azide (~ 1×10^6 cells/100 μL). The cells were then incubated with anti-mouse CD16/CD32 mAb (10 μg/mL) for 5 min on ice and stained with FITC-conjugated anti-mouse CD45R/B220 mAb (10 μg/mL) or PE-conjugated rat anti-mouse CD138 mAb (4 μg/mL) for 30 min on ice. After being washed with and suspended in PBS containing 0.5% BSA and 0.1% sodium azide, the cells were stained with PI (2 μg/mL), and expression of CD45R/B220 and CD138 on viable cells was analyzed by a flow cytometer (Epics XL, Beckman Coulter).

2.7. Determination of apoptotic cells

Apoptotic cells were determined as described previously [19]. Briefly, enriched B cells (~ 2×10^6 total cells), which had been incubated with or without AA-2G for 2 and 4 days in the reculture period, were pooled, washed twice with cold PBS, and fixed with 200 μL of 70% ethanol for 4 h at 4°C. After being washed with PBS, the cells were incubated with ribonuclease A (0.1 mg/mL) in 100 μL of PBS for 30 min at 37 °C. The cells were then washed with and suspended in 1 mL of PBS and incubated with PI (50 μg/mL) for 30 min at room temperature in the dark. The DNA contents of cells were analyzed by a flow cytometer (Epics XL, Beckman Coulter). The cells with a sub-G1 DNA content were regarded as apoptotic cells.

2.8. Determination of DNA synthesis

Enriched B cells, which had been incubated with or without AA-2G for 1–4 days in the reculture period, were pulse-labeled with [^{3}H]thymidine (0.5 μCi/well,

2.5 Ci/mmol) for the last 4 h and harvested on glass fiber filters as described previously [14]. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter. The activity of DNA synthesis was calculated as incorporated [^{3}H]thymidine per 10^5 viable cells. The number of viable cells in parallel cultures was determined by a trypan blue dye-exclusion test.

2.9. Determination of intracellular AA contents

Enriched B cells (0.5–4 × 10^6 viable cells), which had just been prepared or had been incubated for 2 days in the priming period and for 2 and 4 days with or without AA-2G in the reculture period, were pooled, washed three times with cold PBS, and stored at −80°C until analyses. The cells were then suspended in 100 μL of cold 1% metaphosphoric acid and sonicated. The cell homogenates were centrifuged, and the resulting supernatant was subjected to HPLC analysis for estimating AA. The separation of AA was achieved by isocratic elution from an Inertsil ODS-3 column (4.6 i.d. × 250 mm, 5 μm; GL Sciences, Tokyo, Japan) with a guard column of Inertsil ODS-3 (4.6 i.d. × 10 mm, 5 μm; GL Sciences) kept at 40°C with 0.1 M potassium phosphate-phosphoric acid buffer (pH 2.1) containing 10 mg/L of EDTA at a flow rate of 0.7 mL/min as described previously [20]. The absorbance at 240 nm was monitored, and the AA content was determined from the peak area of the sample with reference to the calibration by authentic AA. The intracellular AA contents are expressed as ng per 10^6 viable cells.

2.10. Data Analysis

Results are expressed as means and S.D. of three or four independent experiments. Data in two groups were analyzed by Student's *t*-test. Multiple comparison of the data was done by Dunnett's *t*-test, Bonferroni's test or Dunnett's T3-test. *P* values less than 5% were regarded as significant.

3. Results

3.1. Enhancement by AA-2G of IgM production by B cells stimulated with anti-μ

Murine spleen B cells were primed with anti-μ in the presence of IL-4 and IL-5 and then washed and recultured with or without varying doses (0.063-1 mM) of AA in the presence of IL-4 and IL-5 for 5 days. IgM production was not increased by AA (0.063-0.25 mM), and it was rather decreased by high concentrations of AA (0.5 and 1 mM) (Fig. 1A). In contrast, AA-2G dose-dependently increased IgM production, the greatest enhancement being 150% at concentrations of more than 0.5 mM (Fig. 1A). IgM production was not further potentiated when AA-2G was included in both the priming period and the reculture period (data not shown). Anti-μ-primed B cells produced only a trace amount of IgM in the absence of IL-4 and IL-5 as previously reported by Phillips and Klaus [15] and Weber et al. [16]. The stimulatory effect of AA-2G was not observed in the absence of IL-4 and IL-5 (Fig. 1B). Figure 1C shows a time course for AA-2G-induced increase in IgM production. B cells cultured without AA-2G produced a minimal amount of IgM during the initial 2 days of secondary culture but produced a significant amount of IgM thereafter. The enhancing effect of AA-2G was not observed before day 4 of secondary culture.

3.2. Enhancing effect of AA-2G mediated by released AA

AA-2G is hydrolyzed by cellular α-glucosidase to release AA [11]. To determine whether the enhancing effect of AA-2G on IgM production is mediated by AA, we employed castanospermine, an alkaloidal inhibitor of α-glucosidase [21]. Castanospermine was added to anti-μ-stimulated B cells in the second culture with AA-2G, and the cultures were incubated for 5 days. AA-2G-induced IgM production was almost completely inhibited to the level of cultures without AA-2G by 2 μM castanospermine, which had no effect on IgM production in cultures without AA-2G (Fig. 2A). Changes in AA contents in cells cultured with or without AA-2G were then determined. As shown in Fig. 2B, intracellular AA contents were depleted to undetectable levels at the end of the first culture. Addition of 0.5 mM AA-2G to the cells at the start of the second culture caused a marked increase in intracellular AA contents to more than the initial levels.

3.3. Effects of other stable AA derivatives and antioxidants on IgM production by B cells

AA-2P and AA-2S are also known as stable AA derivatives. AA-2P has been shown to increase collagen synthesis in human fibroblasts, and it has an antiscorbutic activity in guinea pigs and monkeys, whereas AA-2S does not have either activity [22-24]. AA-2P but not AA-2S is appreciably cleaved by lysates of human dermal fibroblasts to release AA [22]. These results suggest that AA-2P but not AA-2S is hydrolyzed by cells in culture and *in vivo*. Effects of both derivatives on IgM production by B cells stimulated with anti-μ, IL-4 and IL-5 were compared with the effect of AA-2G. As shown in Fig. 2C, AA-2P, but not

AA-2S, increased IgM production as markedly as did AA-2G.

Effects of some antioxidants on IgM production by anti-μ-primed B cells were shown in Fig. 2D. Trolox, a cell-permeable derivative of vitamin E, glutathione and uric acid did not enhance IgM production.

3.4. Enhancement by AA-2G of differentiation of anti-μ-stimulated B cells

In order to confirm enhanced differentiation of B cells treated with AA-2G, the number of antibody-secreting cells in cultures treated with or without AA-2G for 4.5 days in the reculture period was determined by the ELISPOT assay, and the results are expressed as number of spots per reculture. Treatment with 0.5 mM AA-2G resulted in 160% increase in the number of IgM-secreting cells (Fig. 3A). It is known that differentiation of naive B cells to plasma cells is accompanied by an increase in CD138 expression and by a decrease in CD45R/B220 expression [25,26]. B cells treated with or without AA-2G for 4 days in the second culture were subjected to analyses of expression of both cell-surface antigens by a flow cytometer. AA-2G significantly increased the percent of $CD138^+$ cells and the percent of $CD45R/B220^-$ cells (Fig. 3B and C).

3.5. Attenuation by AA-2G of the decrease in viable cell number

We noticed during the ELISPOT assay in the experiments for which results are shown in Figure 3A that the viable cell number in cultures treated with AA-2G was larger than that in cultures without AA-2G: an approximately 70% increase in the former (Fig. 3A). The increase in viable cell number may contribute to enhanced IgM production. The time course for changes in viable cell

number in cultures treated with and without AA-2G during the second culture was then determined. The number of viable cells in cultures without AA-2G progressively decreased with incubation time even in the presence of IL-4 and IL-5, but the decrease was significantly attenuated by AA-2G (Fig. 4A). In order to elucidate the mechanism responsible for the slower decrease in cell number, we next determined the proliferation and apoptosis of B cells incubated with or without AA-2G in the second culture. Cell proliferation was determined by [^{3}H]thymidine uptake, expressed as the amount of [^{3}H]thymidine incorporated per 10^5 viable cells. DNA synthesis in cultures without AA-2G decreased more sharply during the second culture than the number of viable cells did (Fig. 4B and A). AA-2G caused a slight but reproducible inhibition of the decrease in DNA synthesis on day 2 of the second culture (Fig. 4B). Figure 4C and D shows the flow cytometric analysis of cells treated with or without AA-2G for 2 and 4 days. The DNA of cells fixed in ethanol was stained with PI. Apoptotic cells, those with a fractional DNA content, as indicated by bars, were decreased on day 4 of the second culture by treatment with AA-2G.

4. Discussion

Our results presented here demonstrated that AA-2G added in the reculture period promoted IL-4- and IL-5-dependent differentiation of anti-μ-primed B cells: AA-2G increased the number of IgM-secreting cells, $CD138^+$ cells and $CD45R/B220^-$ cells, resulting in augmentation of IgM production. Those effects of AA-2G may be mediated by AA continuously released from AA-2G through the action of α-glucosidase because AA-2G-enhanced IgM production was suppressed to the level of IgM production in cells incubated without AA-2G by

the α-glucosidase inhibitor castanospermine. Indeed, intracellular levels of AA in B cells, which was depleted for 2 days during the priming period, were remarkably increased in the reculture period by addition of AA-2G. The level of intracellular AA-2G was undetectable (less than 17 ng/10^6 cells) at 2 and 4 days after addition (our unpublished data), suggesting that AA-2G is not efficiently transported into the cells. The time course for depletion of intracellular AA is similar to the time courses reported for murine T-cell-depleted splenocytes and human tonsilar B cells in culture [2,27]. Since α-glucosidase activity in the medium supplemented with 10% fetal bovine serum is very low for hydrolysis of AA-2G [22] , the conversion of AA-2G to AA may be catalyzed by cellular α-glucosidase of B lymphocytes. In contrast to AA-2G, single addition of AA in the reculture period of B cells did not increase IgM production due to instability of the vitamin in the culture medium [22]. Previous studies have shown that AA-2G as well as 2-*O*-octadecylascorbic acid, AA-2P and AA-2S have a scavenging activity for radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical without being hydrolyzed to AA, although the reaction of the AA derivatives with radicals is much slower than that of AA with radicals [28-30]. However, since AA-2G-promoted IgM production was almost completely inhibited by castanospermine (Fig. 2A) and since AA-2S with a radical scavenging activity did not increase IgM production (Fig. 2C), it seems likely that the contribution of radical-scavenging activity of AA-2G itself to AA-2G-induced augmentation of IgM production is minimal, if any.

Castanospermine is a potent competitive inhibitor of α- and β-glucosidases, but also inhibits the glycoprotein processing glucosidases glucosidase I and glucosidase II [31,32]. Castanospermine causes the change in oligosaccharide

structure of glycoproteins to the immature type by preventing glucose trimming from newly synthesized glycoproteins. Thus, cells treated with castanosperrmine occasionally have fewer receptors at the cell surface or show impaired signal transduction of receptors including IL-2 receptor [32,33]. However, IgM production by IL-4- and IL-5-stimulated B cells primed with anti-μ was not inhibited by castanospermine (Fig. 2A), and to our knowledge, impared expression, binding ability and signal transduction of IL-4 and IL-5 receptors have not been reported in cells incubated with castanospermine. Therefore, it is unlikely that inhibition of AA-2G-induced IgM production by castanospermine was caused by its inhibition of the glycoprotein processing glucosidases.

The antioxidants Trolox, glutathione, and uric acid were ineffective in enhancing IgM production. These data suggest that the antioxidant activity is not the mechanism involved in AA-2G-induced production of IgM. However, the promoting effect of AA-2G was not observed before day 4 of secondary culture (Fig. 1C). We cannot rule out the possibility that activities of those antioxidants was not maintained during the whole culture period.

The effect of AA-2G on differentiation of B cells was not further potentiated even when AA-2G was included in both the priming period and the reculture period. This implies that the initial AA levels present in B cells at the start of priming are high enough to elicit activity of AA if any or that AA has no important function in priming. Purified B cells contain millimolar concentrations of AA, which are much higher than those in plasma (50–150 μM), but the levels of intracellular AA decrease during cell culture [2]. The initial intracellular concentration of AA is maintained by exogenous AA added to cultures repeatedly at 12-hr intervals but not by single addition of AA because of its instability in the culture medium [2]. Single addition of AA-2G, however, is sufficient to restore

and maintain the initial intracellular levels of AA for several days as shown in this study and other studies [27,34]. Thus, AA-2G might be a useful tool for investing the physiological role of AA in long culture systems *in vitro*.

When a value of reported cell volume of resting mouse B cells (100 μm^3) is used [35], the AA concentration in the cells at 0 h in Fig. 2B is calculated to be 2.7 mM that is not so different from the AA concentration (about 1.5 mM) in human B cells [2]. The intracellular concentration of AA is calculated to be 16 mM at 4 and 6 days after addition of AA-2G that is much greater than the initial concentration. Thus, our results suggest that antidoby production by B cells is augmented by AA of concentrations higher than the normal concentration. AA-2G may be helpful in increasing concentrations of AA in B cells.

AA-2G prevented the gradual decrease in the number of viable cells that occurred in anti-μ-stimulated B cell cultures even in the presence of IL-4 and IL-5. The resulting more viable cells in AA-2G-treated cultures (approximate 70% more cells) than in untreated cultures, however, can not fully explain the AA-2G-induced increase in number of IgM-secreting cells (160%). Thus, AA-2G-induced differentiation of B cells into plasma cells may contribute to the difference (90% increase) between the two values. The mechanism involved in AA-2G-induced B cell differentiation remains to be determined. In this connection, it is noteworthy that AA has increased nuclear binding of the transcription factor AP-1 [36,37]. AP-1 has been shown to positively regulate expression of B-lymphocyte-induced maturation protein 1, one of key transcription factors in B cell terminal differentiation, and differentiation of activated B cells [38,39]. We are presently attempting to determine whether B-lymphocyte-induced maturation protein 1 expression in B cells is regulated by AA-2G. As for AA-2G-induced attenuation of the decrease in viable cell number,

our results suggest that this effect is at least partly due to both the inhibition of apoptosis and enhancement of DNA synthesis. An anti-apoptotic effect of AA has been reported in several types of cells, including lymphocytes (40-42), but our results are the first report on ascorbate-induced protection of B cells from apoptosis.

In conclusion, the results of this study demonstrated that AA released from AA-2G enhanced IgM production by B cells primed with anti-μ in the presence of IL-4 and IL-5 and suggested that its effect is caused through promoting the differentiation of B cells to plasma cells and attenuating the gradual decrease in the number of viable cells.

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Figure legends

Figure 1. Enhancement by AA-2G, but not by AA, of IL-4- and IL-5-dependent IgM production by B cells primed with anti-μ. B cells were primed with anti-μ for 2 days in the presence (A–C) or absence (B) of IL-4 and IL-5 and then washed and recultured with the indicated doses of AA or AA-2G for 5 days (A,B) or with or without 0.5 mM AA-2G for the indicated days (C) in the presence (A–C) or absence (B) of IL-4 and IL-5. IgM in the culture supernatants was measured by an ELISA. The data are means ± S.D. or means and S.D. of three independent experiments. Values that are significantly different from those of the respective control cultures incubated without AA-2G or AA are indicated by $*p < 0.05$, $***p < 0.001$ (Dunnett's *t*-test) (A) and $**p < 0.01$ (Student's *t*-test) (B,C).

Figure 2. Enhancing effect of AA-2G mediated by released AA. B cells were primed with anti-μ for 2 days in the presence of IL-4 and IL-5 and then washed and recultured with or without AA-2G (0.5 mM), other stable AA derivatives (0.5 mM) or the indicated concentrations of antioxidants for 5 days (A,C,D) or for 2 and 4 days (B) in the presence of IL-4 and IL-5. (A) Inhibition of AA-2G-induced enhancement of IgM production in B cells by the α-glucosidase inhibitor castanospermine. (B) Changes in AA contents in the incubated B cells. AA contents in 1% metaphosphoric acid extracts of pooled cells were estimated by HPLC analysis. (C) Effects of other stable AA derivatives on IgM production in B cells. (D) Effects of antioxidants on IgM production in B cells. Trolox was dissolved in dimethyl sulfoxide (DMSO) and added to the medium at the final DMSO concentration of 0.05%. IgM in the culture supernatants was measured by an ELISA. The data are means and S.D. or means ± S.D. of three (A,B,D) or four

(C) independent experiments. (A) ***$p < 0.001$, as compared with the values of control cultures incubated without AA-2G and castanospermine (Bonferroni's test). $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, as compared with the values of cultures incubated with AA-2G alone (Bonferroni's test). (B) **$p < 0.01$, as compared with the values of zero time cells (Dunnett's T3-test). (C) *$p < 0.05$, as compared with the values of control cultures incubated without AA derivatives (Dunnett's *t*-test). (D) **$p < 0.01$, ***$p < 0.001$, as compared with the values of control cultures incubated without antioxidants (Dunnett's *t*-test).

Figure 3. Increase by AA-2G in the number of IgM-secreting cells, $CD138^+$ cells and $CD45R/B220^-$ cells elicited by anti-μ, IL-4 and IL-5. B cells were primed with anti-μ for 2 days in the presence of IL-4 and IL-5 and then washed and recultured with or without 0.5 mM AA-2G for 4.5 days (A) or 4 days (B,C) in the presence of IL-4 and IL-5. (A) The number of IgM-secreting cells and the number of viable cells were measured by an ELISPOT assay and the trypan blue dye-exclusion test, respectively. (B,C) The cells were stained with PE-conjugated anti-CD138 mAb and PI (B) or FITC-conjugated anti-CD45R/B220 mAb and PI (C) and analyzed by a flow cytometer. The data are means and S.D. of three independent experiments (A–C). Histograms (B,C) are representative of three independent experiments. *$p < 0.05$, ***$p < 0.001$, as compared with the values of control cultures incubated without AA-2G (Student's *t*-test).

Figure 4. Effects of AA-2G on viable cell number, DNA synthesis and apoptosis in B cells stimulated with anti-μ, IL-4 and IL-5. B cells were primed with anti-μ for 2 days in the presence of IL-4 and IL-5 and then washed and recultured with or without 0.5 mM AA-2G for the indicated days in the presence of IL-4 and IL-5.

(A) Attenuation by AA-2G of the gradual decrease in viable cell number during the reculture period. The number of viable cells was determined by the trypan blue dye-exclusion test. (B) Enhancement by AA-2G of DNA synthesis in B cells stimulated with anti-μ, IL-4 and IL-5. Cells were labeled with [^{3}H]thymidine for the last 4 h. The number of viable cells in parallel cultures was determined by the trypan blue dye-exclusion test. (C,D) Inhibition of apoptosis by AA-2G in B cells stimulated with anti-μ, IL-4 and IL-5. Cells that had been fixed with 70% ethanol and incubated with ribonuclease A were stained with PI, and the DNA contents of cells were analyzed by a flow cytometer. The cells with a sub-G1 DNA content were regarded as apoptotic cells. The data are means ± S.D. or means and S.D. of three independent experiments. Histograms (C) are representative of three independent experiments. Values that are significantly different from the respective control cultures incubated without AA-2G are indicated by $*p < 0.05$, $**p < 0.01$ (Student's *t*-test).

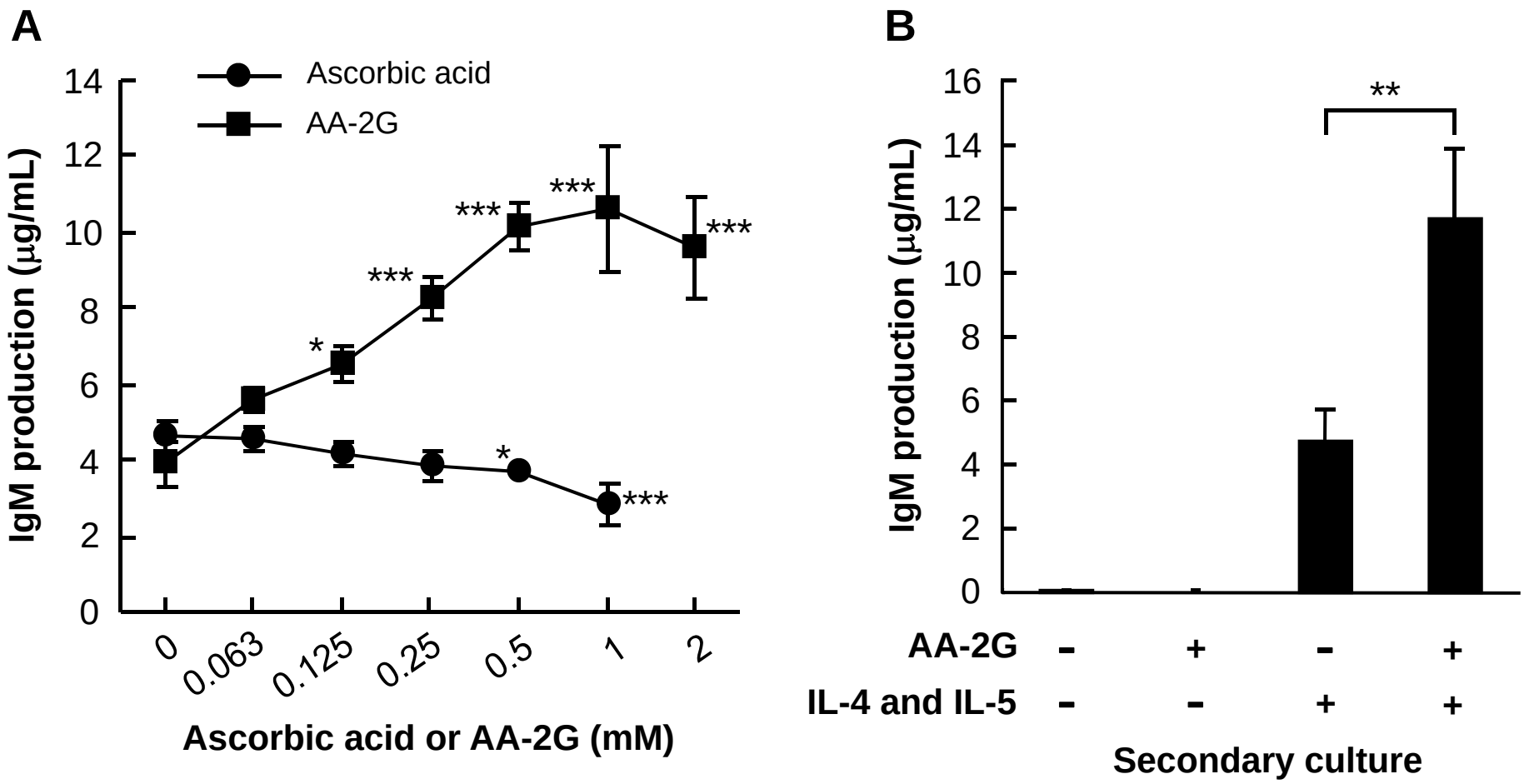

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Fig. 1A & 1B

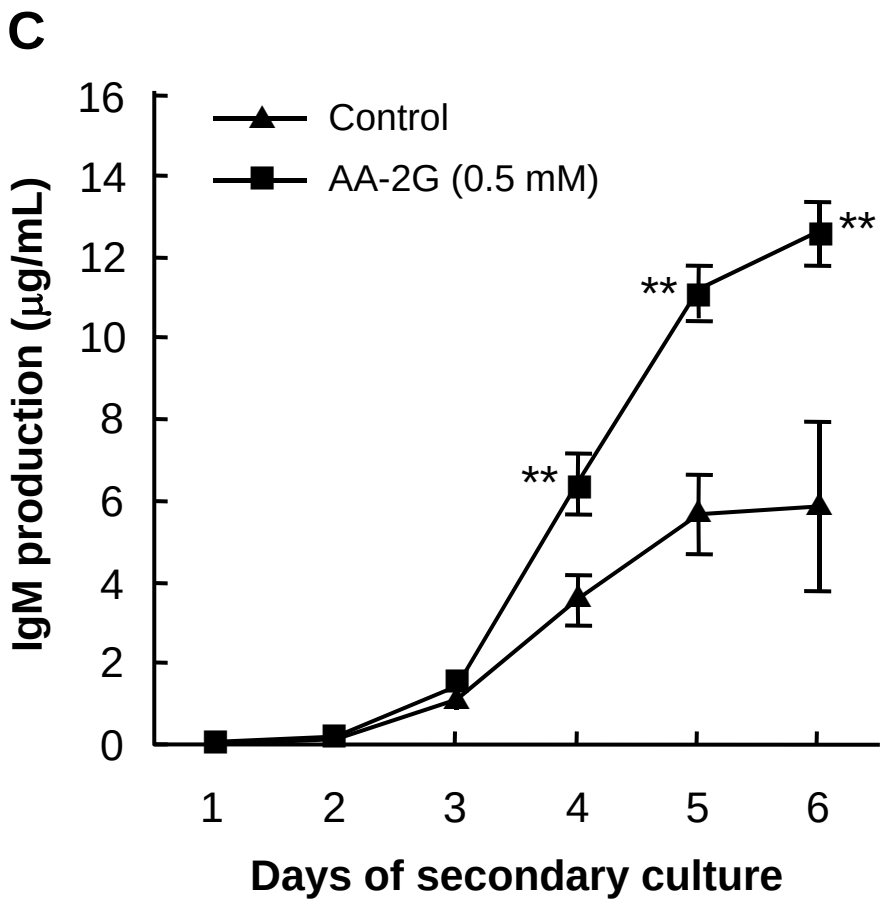

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Fig. 1C

A

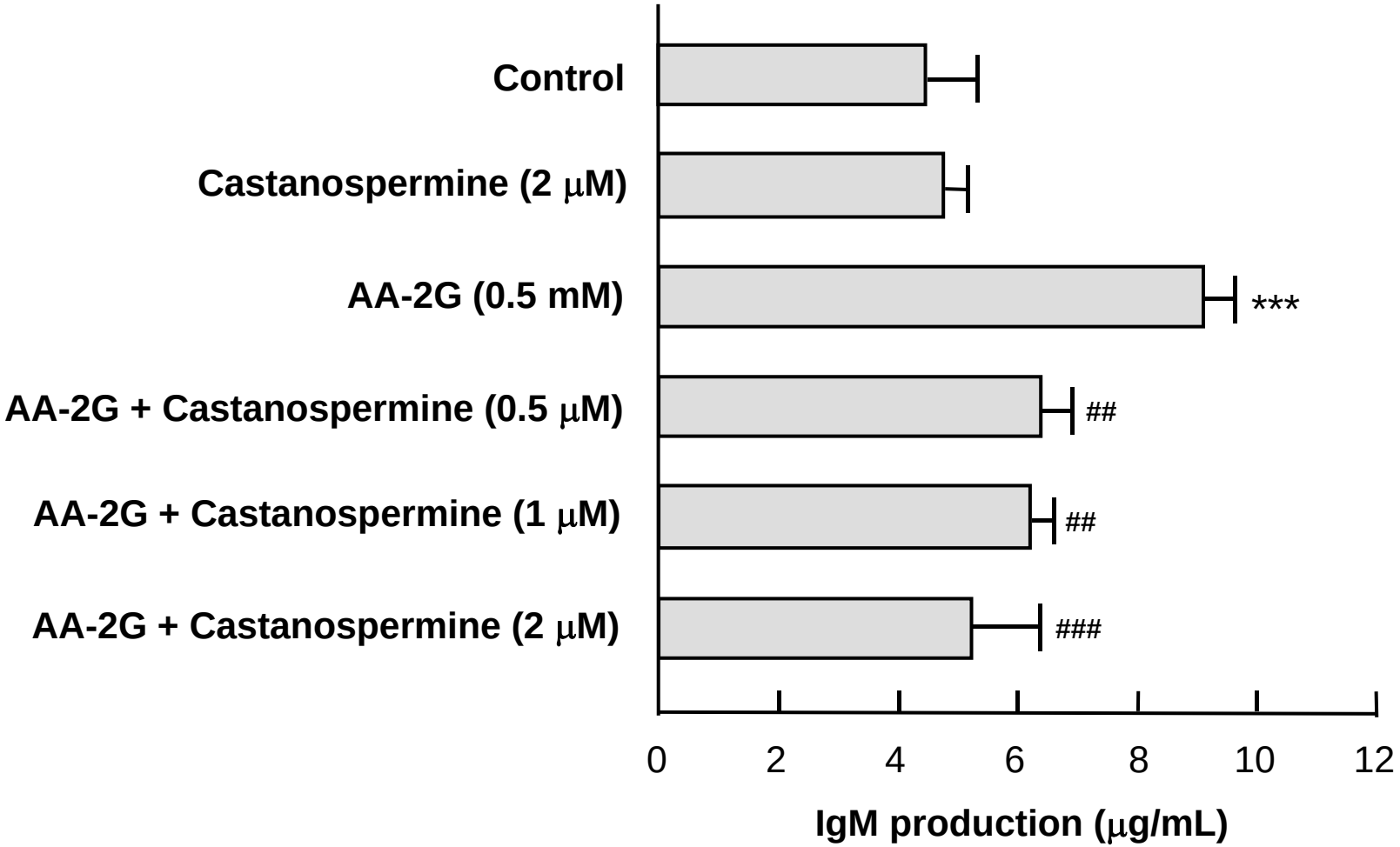

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Fig. 2A

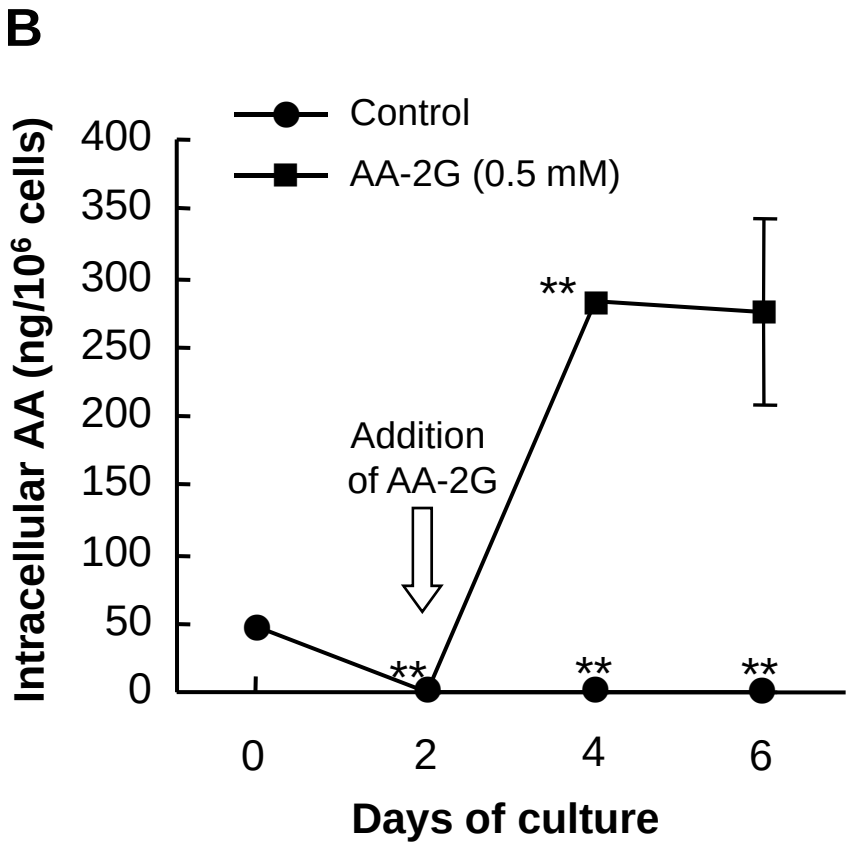

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Fig. 2B

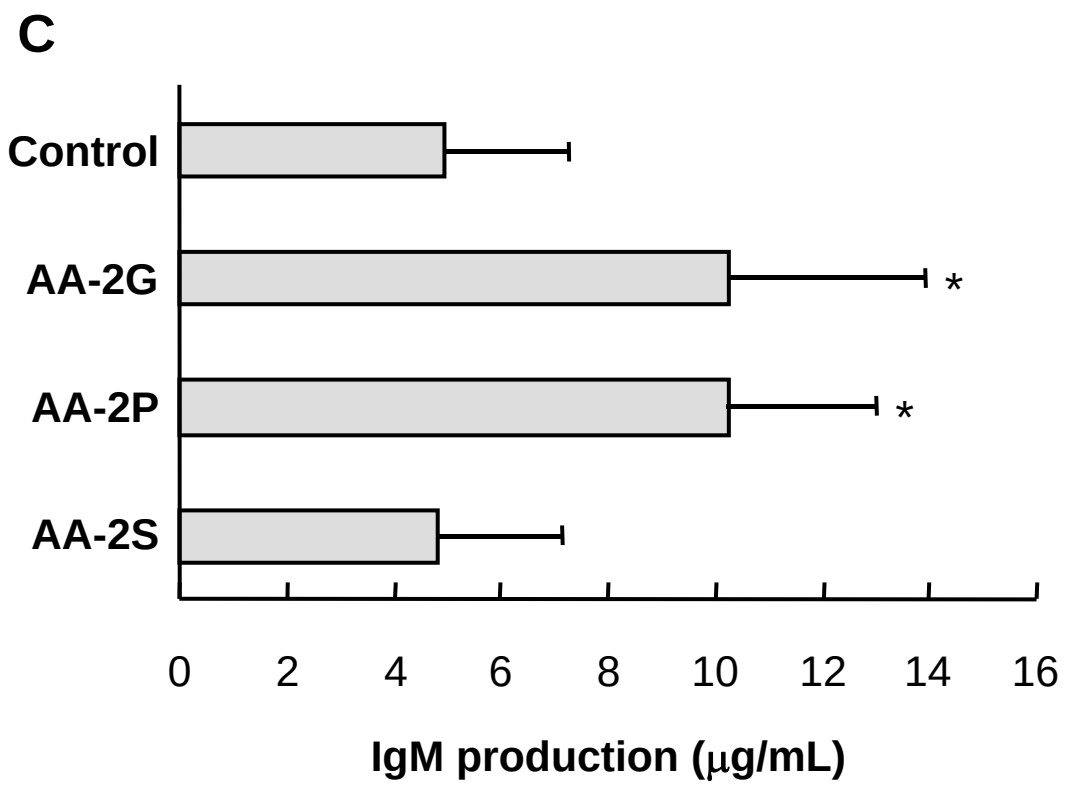

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Fig. 2C

D

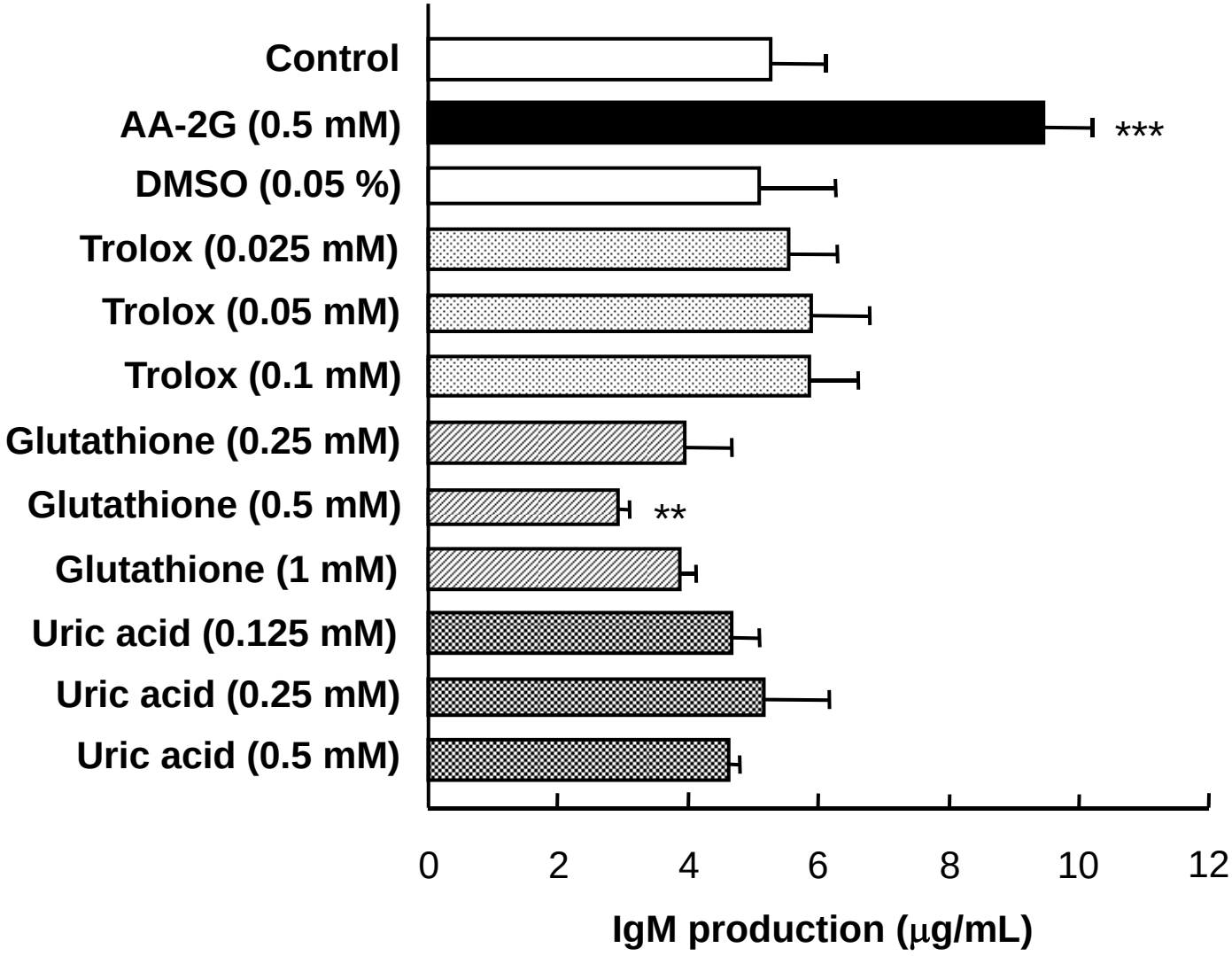

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Fig. 2D

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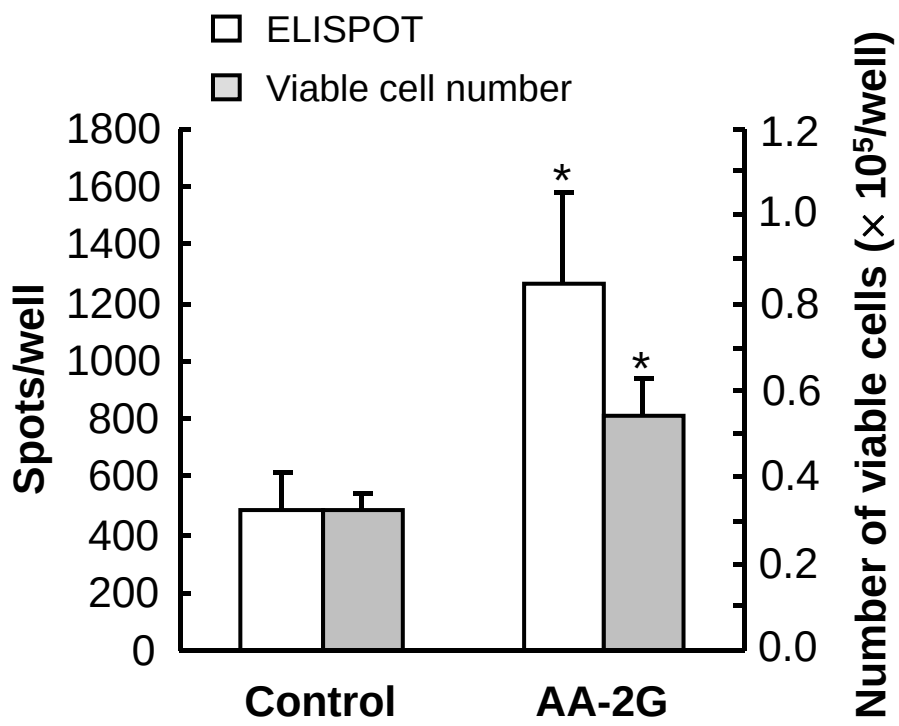

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Fig. 3A

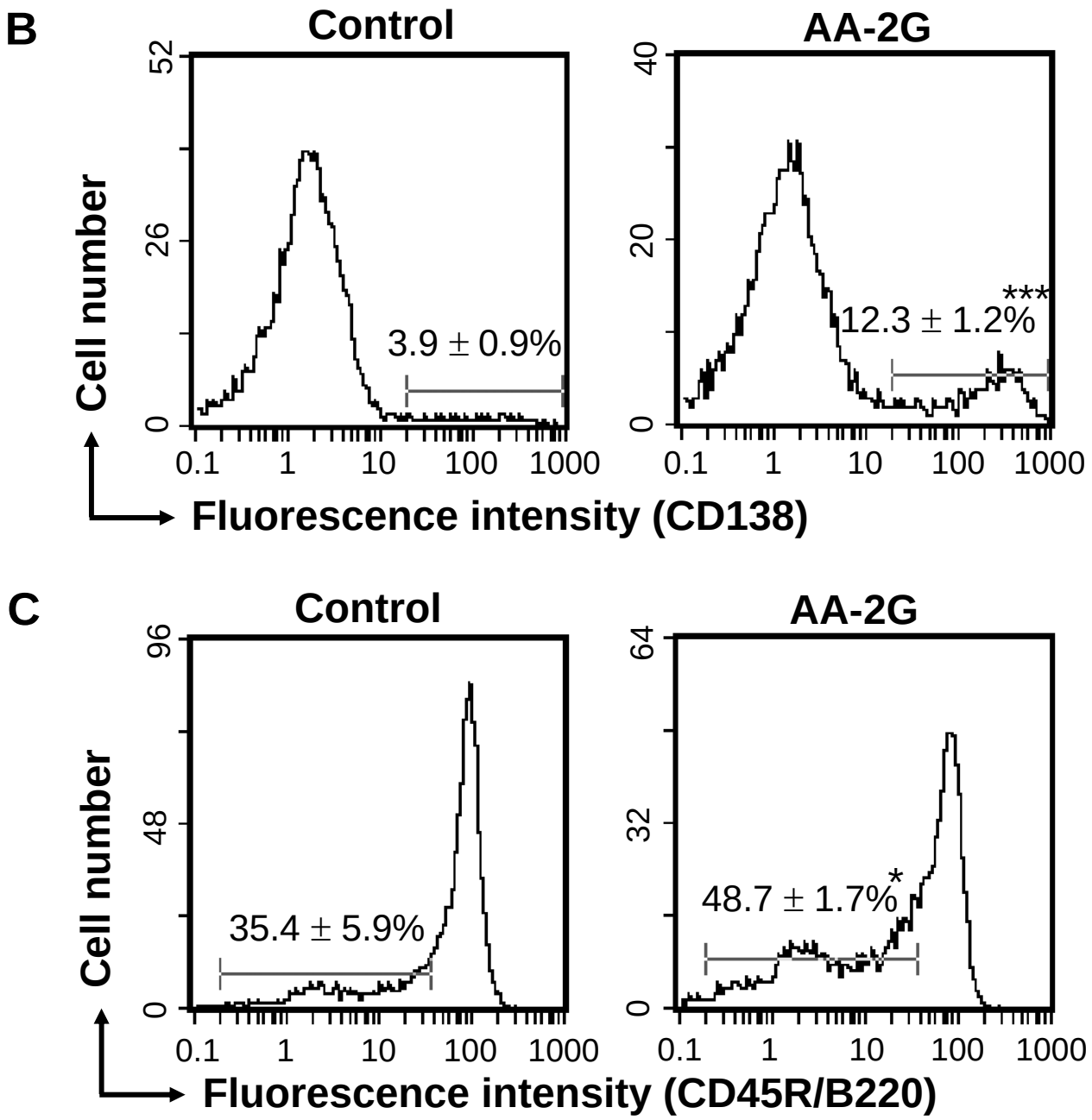

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Fig. 3B & 3C

A

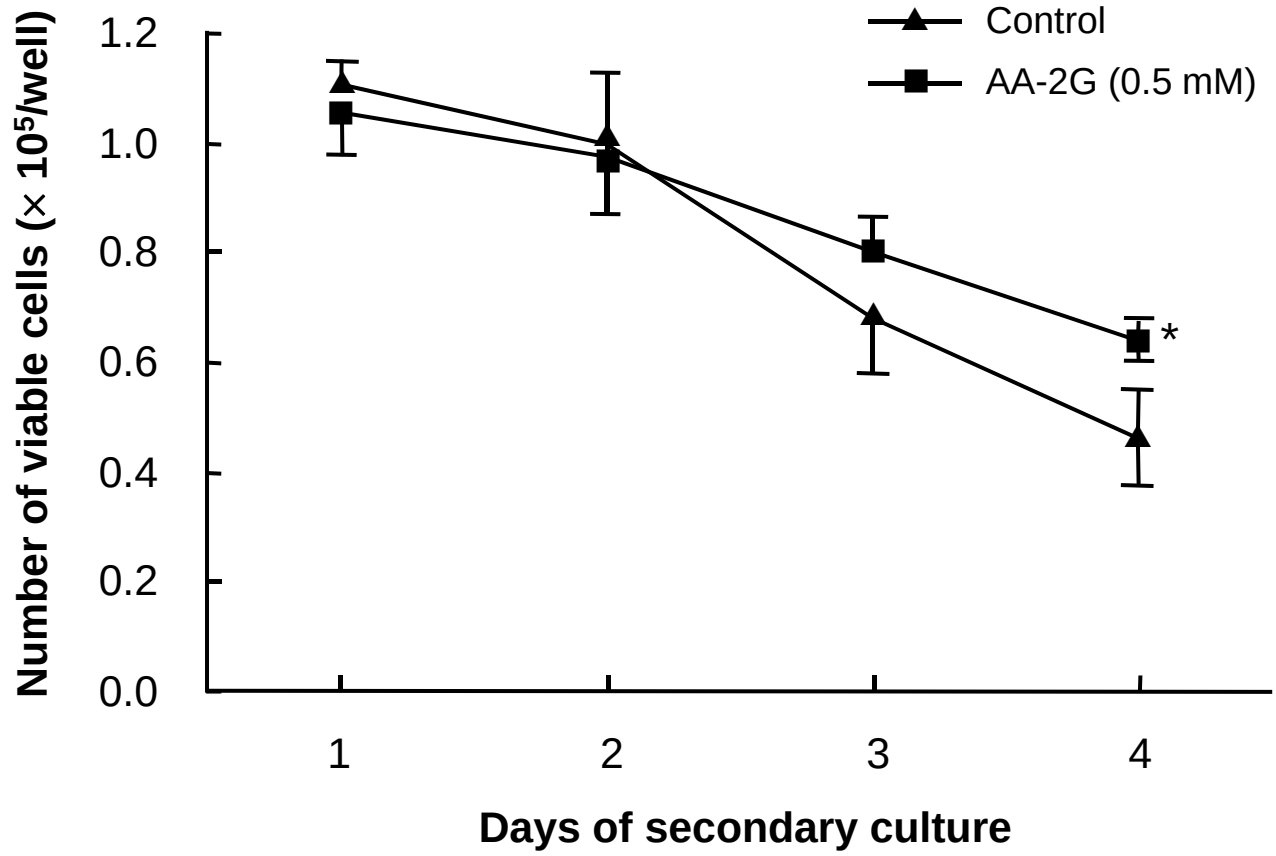

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Fig. 4A

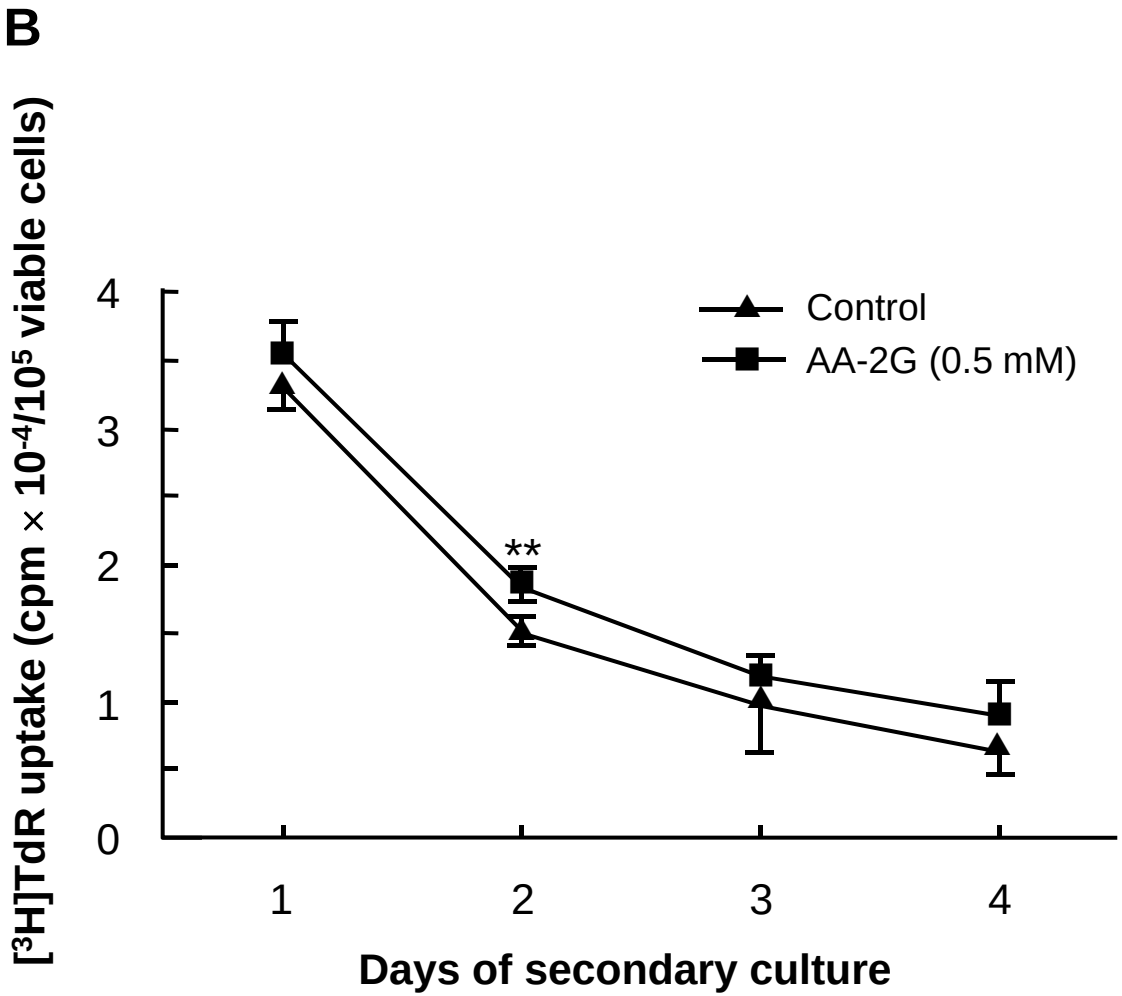

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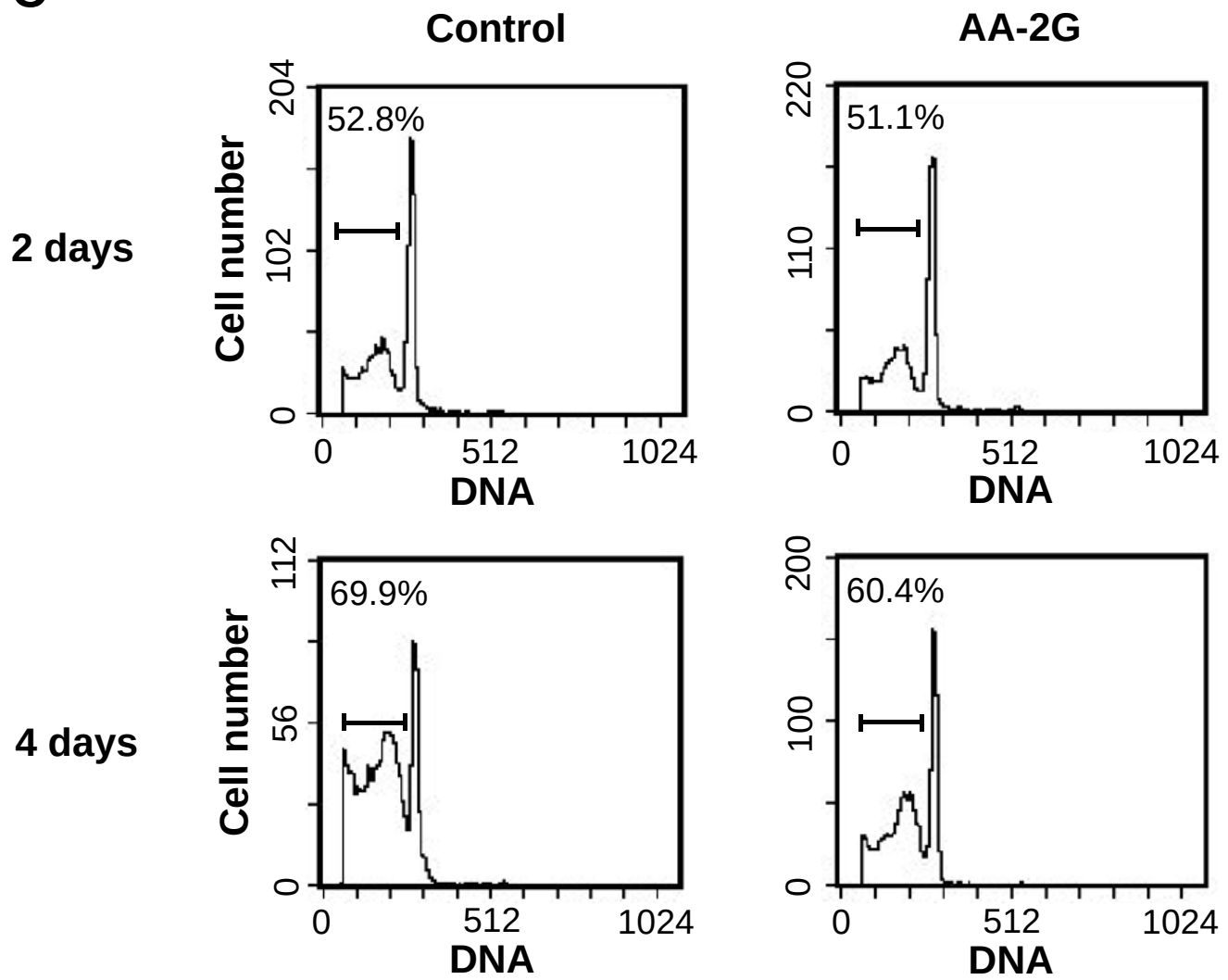
Fig. 4B

C



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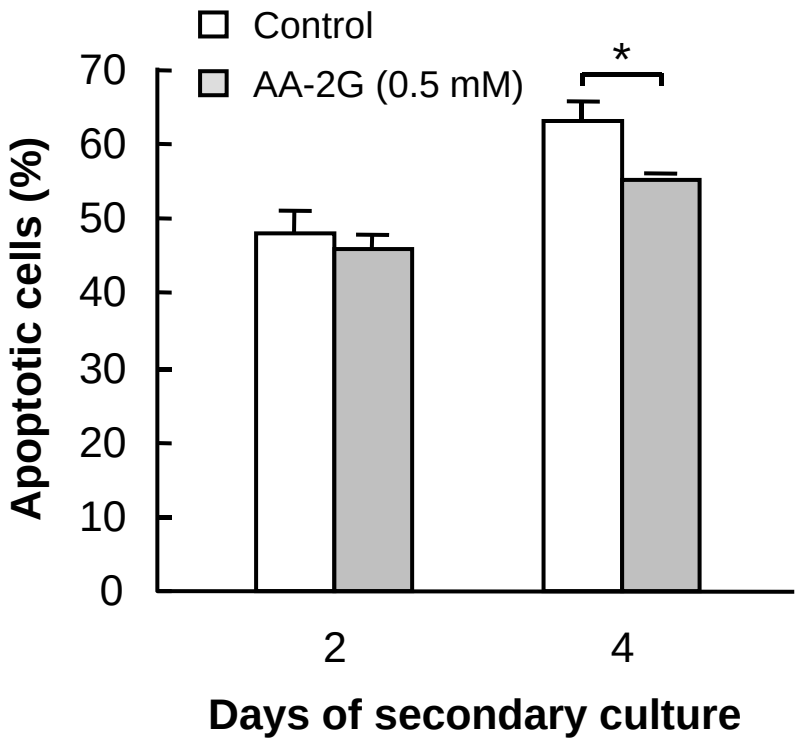

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Fig. 4D