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Macrophage Recognition of Cells with Elevated Calcium Is Mediated by Carbohydrate Chains of CD43

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ABSTRACT. **Macrophages remove deteriorating cells (those undergoing apoptosis and oxidation) via poly-*N*-acetyllactosaminyl chains on CD43 caps, a major cell-surface glycoprotein. Unusually high intracellular calcium levels are also deteriorating for cells and tissue. Here we artificially elevated calcium levels in cells and examined the mechanism by which this elevation was resolved by macrophages. Results showed that treatment with the calcium ionophore A23187 and ionomycin induces capping of CD43 on Jurkat cells, which are subsequently recognized and phagocytosed by macrophages, indicating that macrophages regard cells with elevated calcium as targets for removal. Further tests showed that A23187- and ionomycin-treated Jurkat cells did not induce apoptotic changes such as DNA fragmentation or phosphatidylserine expression, indicating that these cells were removed despite still being viable. Jurkat cells pretreated with anti-CD43 antibody or those with poly-*N*-acetyllactosaminyl chains containing oligosaccharides inhibited macrophage binding, indicating that macrophages recognize the poly-*N*-acetyllactosaminyl chains on CD43. Binding was also inhibited by treating macrophages with anti-nucleolin antibody, indicating that recognition occurs through nucleolin, a cell-surface receptor. Further, nucleolin-transfected HEK293 cells bound A23187-treated cells, and this binding was inhibited by in the presence of oligosaccharides. Taken together, these results show that elevated calcium levels induce CD43 capping, and macrophages remove the cells if their nucleolin receptors can bind to the poly-*N*-acetyllactosaminyl chains of capped CD43.**

Key words: elevated calcium, CD43, cytoskeleton, macrophage, nucleolin

Introduction

Deteriorating cells, including those undergoing apoptosis, oxidation, and senescence are rapidly recognized and ingested by macrophages before they collapse and release harmful intracellular contents (Aderem and Underhill, 1999; Hanayama *et al.*, 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000). If these cells are not removed, the contents injure surrounding tissue and induce abnormal autoimmune responses, potentially triggering any of several functional disorders (Aderem and Underhill, 1999; Hanayama *et al.*, 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000). Macrophages also suppress inflammation by inhibiting pro-inflammatory cytokine production and releasing anti-inflammatory cytokines (Fadok *et al.*, 2001; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000) and are therefore critical for maintaining homeostasis and healthy tissue by safely clearing deteriorating cells and reducing inflammation. Hypercalcemia and unusually high intracellular calcium levels are also deteriorating condition for cells and tissue, because unusually high intracellular calcium levels disrupt host homeostasis (Legrand, 2011; Kim *et al.*, 2002; McConkey *et al.*, 1989b, 1989a). However, physiological defense mechanism for unusual calcium-elevation was poorly understood. Present study investigated that the removal mechanism of calcium-elevated cells by macrophages.

Successful removal of deteriorating cells (those undergoing apoptosis, oxidation, and senescence) depends on the interaction between their cell-surface ligands and corresponding macrophage receptors, several of which have been reported, including phosphatidylserine (PS) and carbohy-

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Abbreviations: Anti-NUC295, an antibody against amino acid residues 295-302 of nucleolin; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acidtetrakis (acetoxymethyl) ester; BSA, Bovine serum albumin; DPBS(–), Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline; FBS, Fetal bovine serum; FSC, Forward scatter; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MFG-E8, Milk fat globule epidermal growth-factor 8; RPMI 1640-HEPES, RPMI 1640 medium buffered with 20 mM HEPES pH 7.2; PS, Phosphatidylserine; SLE, Systemic lupus erytheatosus; SSC, Side scatter; TUNEL, TdT-mediated dUTP-biotin Nick End Labeling; Z-VAD-fmk, Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone.

drate chains (Savill *et al.*, 1993; Savill and Fadok, 2000; Fadok *et al.*, 2001; Platt *et al.*, 1998; Eda *et al.*, 2004). PS is a well-known ligand located on deteriorating cells, and several receptors for it have been found on macrophages, including CD36 (Rigotti *et al.*, 1995), CD68 (Sambrano and Steinberg, 1995), lectin-like oxidized low-density lipoprotein receptor 1 (Oka *et al.*, 1998), Tim-4 (Miyanishi *et al.*, 2007), and stabillin-2 (Park *et al.*, 2008). Additionally, conserved motifs on deteriorating cells are recognized by the mannose receptor (Aderem and Underhill, 1999; Ogden *et al.*, 2001).

Although the mechanism by which macrophages recognize carbohydrate ligands on deteriorating cells is not fully understood, our previous studies have shed considerable light on the process (Ando *et al.*, 2000; Eda *et al.*, 2004; Miki *et al.*, 2009; Beppu *et al.*, 1996a). Deteriorating cells undergo capping by the membrane glycoprotein CD43, and macrophages recognize these cells through the cluster of poly-*N*-acetyllactosaminyl chains that become available when CD43 caps are formed (Eda *et al.*, 2004; Miki *et al.*, 2009). In a previous study, we identified nucleolin, a multifunctional shuttle protein, as the macrophage receptor for these carbohydrate chains on apoptotic and oxidized cells (Hirano *et al.*, 2005; Miki *et al.*, 2009).

CD43 capping is thought to occur as follows: CD43 normally binds the cytoskeleton protein actin (Fukuda, 1996; Ostberg *et al.*, 1998), and capping occurs when this bond is broken by caspases in the early stages of apoptosis (Kondo *et al.*, 1997; Eda *et al.*, 2004; Miki *et al.*, 2009). Indeed, CD43 capping has been induced artificially using inhibitors that block actin and microtubule polymerization (Oguri *et al.*, 2012; Seveau *et al.*, 1997). CD43 may be conjugated to several cytoskeleton proteins, and disrupting the actin bond could cause active movement of other cytoskeleton proteins that then induce cap formation.

Unusual intracellular calcium elevation is an abnormality observed in many pathological conditions including common variable immunodeficiency, systemic lupus erytheatosus (SLE), Alzheimer's disease, and brain trauma, as well as conditions resulting from toxins such as kainite and maitotoxin (Feske, 2007; Liossis *et al.*, 1996; Saido *et al.*, 1994; Saito *et al.*, 1993; Tsuji *et al.*, 1998; Lee *et al.*, 1991; Hajimohammadreza *et al.*, 1997; Saatman *et al.*, 1996; Nakagawa *et al.*, 2000). Calcium is also known to induce active movement of cytoskeleton proteins that lead to a variety of membrane-related processes, including exocytosis, phagocytosis, membrane deformation, and capping (Oertner and Matus, 2005; Bourguignon and Pressman, 1983). CD43 caps may therefore form in cells with elevated calcium levels, resulting in recognition and phagocytosis by macrophages. Here, we artificially elevated levels in Jurkat cells with A23187 and ionomycin, calcium ionophores, and tested whether or not elevated calcium induces CD43 capping and subsequent removal by macrophages via the carbohydrate-mediated mechanism.

Materials and Methods

Materials

A23187, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acidtetrakis (acetoxymethyl) ester (BAPTA-AM), calcein-AM, ionomycin, PKH 26 red, and PKH67 green fluorescent cell linker kits were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD43 mouse monoclonal antibody (clone DF-T1) and control-mouse IgG1 were obtained from DAKO (Glostrup, Denmark). Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk) was obtained from the Peptide Institute (Osaka, Japan). Anti-nucleolin antibody (anti-NUC295, an antibody against the amino acid residues 295–302 of nucleolin) was raised in rabbits and affinity purified similar to methods previously described (Eda *et al.*, 2001).

Oligosaccharides from human erythrocyte membrane glycoprotein (mainly band 3 glycoprotein) were prepared by hydrazinolysis of defatted human erythrocyte ghosts, as described previously (Beppu *et al.*, 1996b).

Treatment of Jurkat cells with A23187 or ionomycin

Jurkat cells (RIKEN Cell Bank, Tsukuba, Japan) were incubated at 4×10^6 cells/mL in RPMI 1640 medium containing 5% fetal bovine serum (FBS) with A23187 or ionomycin concentrations of 0–2 μM at 37°C in 5% CO_2 atmosphere for 0–12 h.

Binding and phagocytosis assays

Binding and phagocytosis assays were employed as described previously (Eda *et al.*, 2004). Briefly, macrophages were generated using 50 nM phorbol myristate acetate on coverslips to differentiate THP-1 cells (Japanese Cancer Research Resources Bank, Osaka, Japan). A23187- or ionomycin-treated Jurkat cells and macrophage monolayers were co-incubated on coverslips at 37°C for 1 h with gentle shaking. After removing unbound cells, attached cells were fixed, stained, and counted. Data are expressed as the number of bound Jurkat cells/100 macrophages (minimum count of 200 macrophages).

To assay phagocytosis, A23187-treated Jurkat cells were labeled with the fluorescent cell-linker compound PKH67 (green) kit and co-incubated with macrophage monolayers as in the binding assay, after which membranes were stained with the fluorescent cell-linker compound PKH 26 (red) kit. Jurkat cells taken up by macrophages were identified using confocal laser scanning fluorescence microscopy (μ-Radiance; Bio-Rad, Hercules, USA).

Detection of cell-surface CD43

Distribution of CD43 on Jurkat cells was observed as described previously (Eda *et al.*, 2004). Briefly, Jurkat cells were treated with anti-CD43 antibody in RPMI 1640 medium bufferized with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (RPMI 1640-HEPES) with 0.2% bovine serum albumin (BSA) for

30 min. Washed and bound antibodies were detected using Alexa Fluoro-488 conjugated goat anti-mouse IgG (H+L) and then immediately subjected to observation via a fluorescence microscope. Throughout the immunofluorescence staining process, cell suspensions were kept at 0°C to prevent antibody-induced antigen redistribution that may occur at higher temperatures.

Detection of apoptosis in A23187- or ionomycin-treated Jurkat cells

The apoptotic states of Jurkat cells treated with A23187 or ionomycin were assessed by examining DNA fragmentation and PS expression, using commercial assay kits (MEBCYTO Apoptosis Kit and MEBSTAIN Apoptosis Kit Direct, respectively [Medical and Biological Laboratories, Nagoya, Japan]). PS expression was determined by binding fluorescein isothiocyanate-labeled annexin V (FITC-annexin V) to the cells. Briefly, Jurkat cells (2×10^5 cells) suspended in a kit buffer containing FITC-annexin V were incubated at room temperature for 15 min in the dark, in accordance with the manufacture's instruction. The cell suspension was then diluted with another buffer and immediately analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, USA) with CELLQUEST software and gating for forward scatter (FSC) (correlating with the cell volume) and side scatter (SSC) (depending on the inner complexity of the particle) regions of intact Jurkat cells.

DNA fragmentation was assessed using TdT-mediated dUTP-biotin Nick End Labeling (TUNEL). Briefly, Jurkat cells (2×10^6 cells) were fixed with 4% paraformaldehyde at 4°C for 0.5 h. After washing with 0.2% BSA-containing DPBS(–) (0.2% BSA-DPBS[–]), the cells were permeabilized using 70% ethanol at –20°C for 0.5 h. After washing with 0.2% BSA-DPBS(–), the DNA nick ends of cells were labeled by treatment with a kit buffer containing TdT reaction reagent at 37°C for 1 h in accordance with the manufacturer's instructions. Cells were then washed using 0.2% BSA-DPBS(–) and immediately subjected to flow cytometric analysis (FACSCalibur) with gating for FSC and SSC regions of intact Jurkat cells.

Calcein-AM treatment to A23187-treated Jurkat cells for phagocytosis assay

A23187-treated Jurkat cells were incubated with 1 μM of calcein-AM at 37°C for 20 min. The dye was removed by washing, and then the cells were subjected to the phagocytosis assay.

Binding assay for recombinant human nucleolin-expressing HEK 293 cells

Human nucleolin cDNA was transfected into the monolayer of HEK 293 cells (HEK cells; Health Science Research Bank, Osaka, Japan) as previously described (Hirano *et al.*, 2005). A23187-treated Jurkat cells were applied to the HEK monolayer and co-incubated at 37°C for 1 h with gentle shaking. After removing unbound cells, attached cells were fixed, stained, and counted. Data are expressed as the number of bound Jurkat cells per 100 HEK cells (minimum count of 200 cells).

Statistical analysis

The data are presented as the mean±SD of at least three experiments and analyzed using Student's *t*-test.

Results

Treatment with calcium ionophore A23187 induces cell-surface capping of CD43

We observed that while CD43 distribution on the surface of Jurkat cells was uniform on Jurkat cells (Fig. 1A, left panel), distribution was localized in a cap on A23187-treated Jurkat cells (Fig. 1A, right panel). Capping required a minimum of 10 nM of A23187 (Fig. 1B), and the largest number of capped cells was observed after treating with 100 nM for 0.5–1 h (Fig. 1C), declining after 2 h. A similar dosage of A23187 (100 nM) has been shown to increase intracellular calcium levels from 90 nM to >10 μM in Jurkat cells (Sasaki and Hasegawa-Sasaki, 1985). Additionally, calcium EDTA-chelation inhibited the capping of CD43 on A23187-treated cells (Fig. 1D). These results indicate that the elevated intracellular calcium levels of A23187-treated cells led to capping of CD43, and as such, we subsequently used Jurkat cells treated with 100 nM of A23187 for 1 h unless otherwise indicated.

A23187-treated cells were recognized and phagocytosed by macrophages

We compared treatment groups and counted the number of Jurkat cells bound to macrophages as well as the number that were phagocytosed. After treatment with A23187, both the number of bound Jurkat cells (Fig. 2A, left panel) and the number of phagocytosed Jurkat cells (right panel) increased significantly. Notably, EDTA-chelation inhibited this increase (Fig. 2B). Taken together, these results show that A23187-treated cells with elevated calcium were recognized and phagocytosed by macrophages.

Capping of A23187-treated Jurkat cells is independent of apoptosis

We previously reported that capping of CD43 was induced during apoptosis by activated caspases (Eda *et al.*, 2004; Miki *et al.*, 2009). Because A23187 is known to cause apoptosis under certain conditions (Kim *et al.*, 2002; McConkey *et al.*, 1989b, 1989a), A23187-treated cells may also became apoptotic, thereby inducing capping. To eliminate this possibility, we examined the cells for two additional signs of apoptosis (DNA fragmentation and PS expression) and

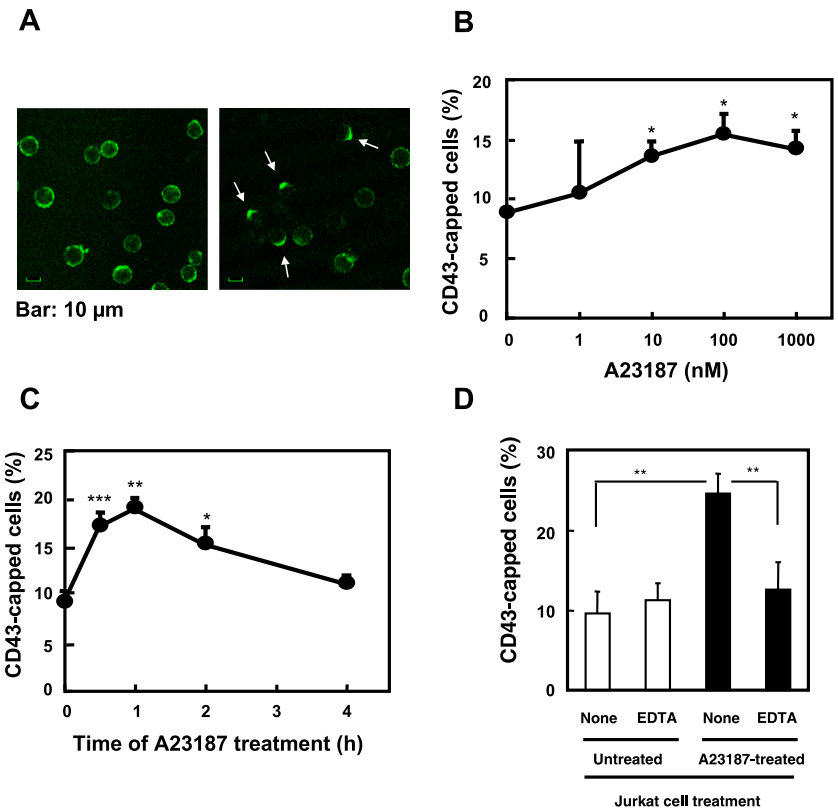

Fig. 1. Treatment with calcium ionophore A23187 induces cell-surface capping of CD43. (A) Distribution of CD43 on Jurkat cells was observed using confocal laser scanning immunofluorescence microscopy (μ-Radiance; Bio-Rad, Hercules, USA). CD43 distribution on untreated Jurkat cells (left panel) and cells treated with 100 nM of A23187 for 1 h (right panel). The arrows indicate capping of CD43. (B and C) Changes in CD43 capping on Jurkat cells as a function of concentration (B) and duration (C) of A23187-treatment. (D) Effect of co-incubation with 2 mM calcium chelator EDTA on CD43 capping on A23187-Jurkat cells. Each point represents the mean±SD of at least triplicate experiments. Statistical analysis was conducted between the control and indicated sample. *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$.

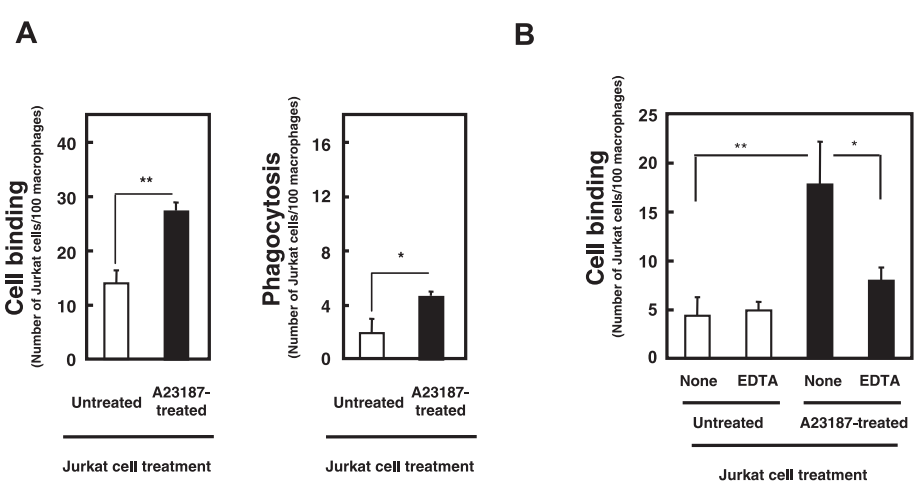

Fig. 2. A23187-treated CD43-capped cells were recognized and phagocytosed by macrophages. (A) Binding (left panel) and phagocytosis (right panel) of A23187-treated and untreated Jurkat cells. (B) Effect of co-incubation with 2 mM calcium chelator EDTA on the recognition of A23187-treated Jurkat cells. Each column represents the mean±SD of at least triplicate experiments. *, $p<0.05$; and **, $p<0.01$.

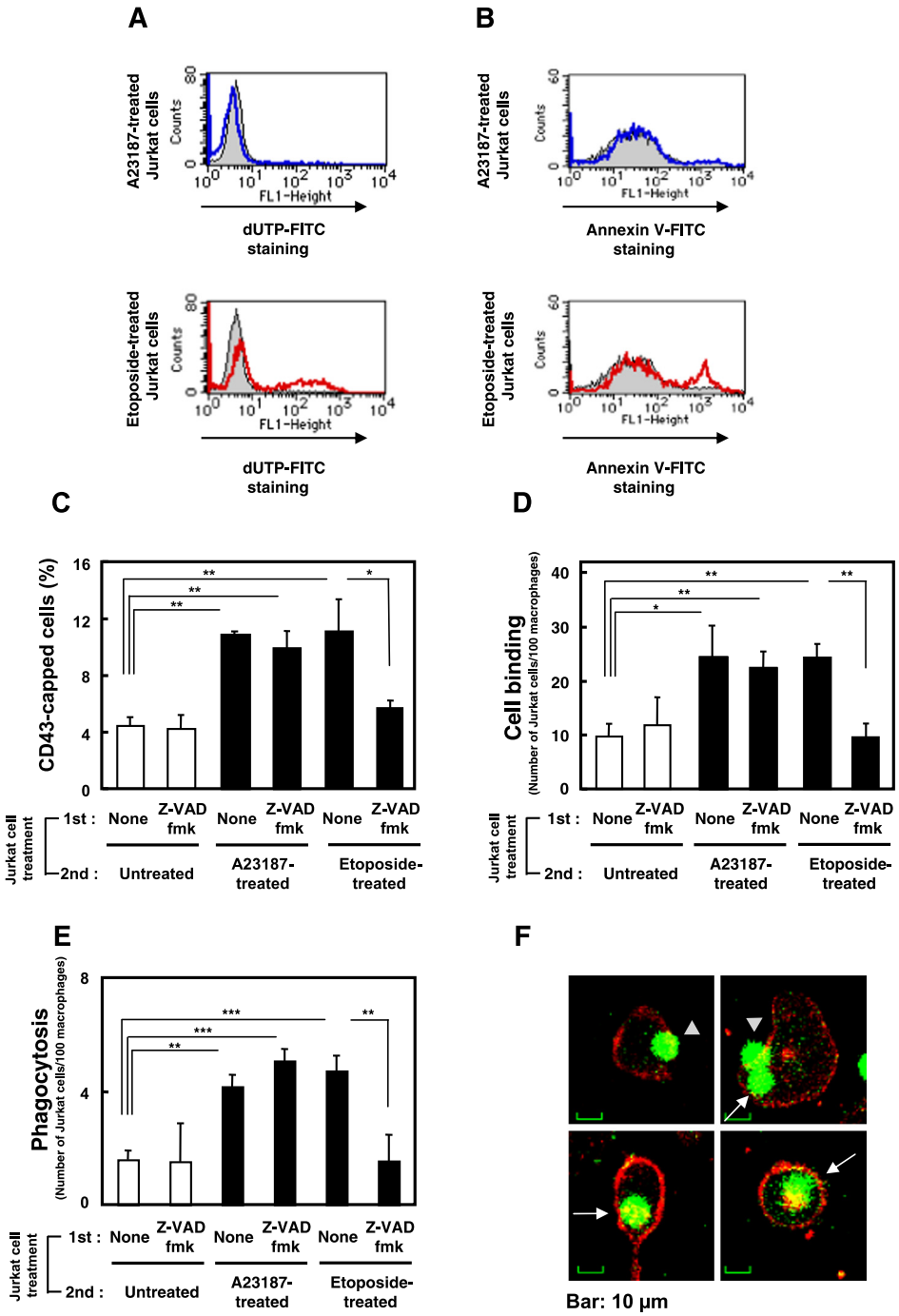

Fig. 3. Capping of A23187-treated Jurkat cells is independent from apoptosis. Measurement of DNA fragmentation (A) and PS expression (B). Jurkat cells were treated with 100 nM A23187 (upper panel, blue line) or 10 μM etoposide (lower panel, red line) at 37°C for 24 h (A) or 4 h (B). Grey shades, fresh Jurkat cells. (C) Effect of caspase inhibitor Z-VAD-fmk on CD43 capping of A23187- or etoposide-treated Jurkat cells. (D) Effect of caspase inhibitor Z-VAD-fmk on recognition of A23187- or etoposide-treated Jurkat cells. (E) Effect of caspase inhibitor Z-VAD-fmk on phagocytosis of A23187- or etoposide-treated Jurkat cells. (F) Confocal images of macrophages ingesting A23187-treated Jurkat cells with calcein-labeling. Jurkat cells were labeled with calcein instead of PKH67. Jurkat cells taken up by macrophages were identified using confocal laser scanning fluorescence microscopy (FV1000D; Olympus, Tokyo, Japan). Green cells, Jurkat cells. Red area, macrophage cell surface. The arrowheads indicate ingesting Jurkat cells; the arrows indicate ingested Jurkat cells. Each column represents the mean±SD of at least triplicate experiments. *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$.

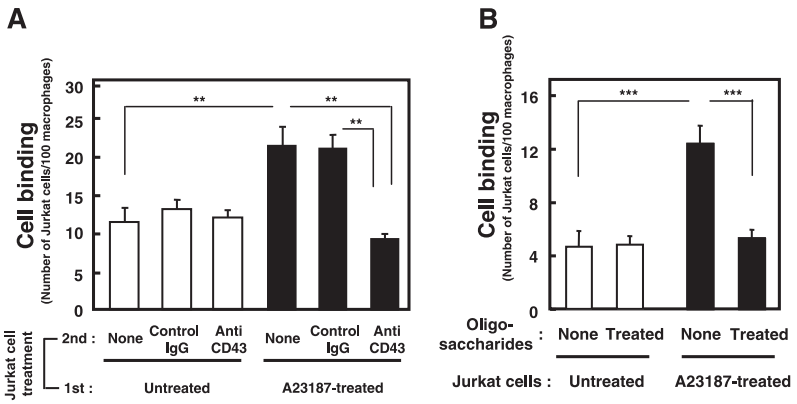

Fig. 4. Role of CD43 and its sugar chains in the recognition of A23817-treated Jurkat cells. (A) Anti-CD43 antibody inhibits binding of macrophages to A23187-treated Jurkat cells. (B) Oligosaccharides prepared from erythrocyte membranes inhibit the binding of macrophages to A23187-treated Jurkat cells. Each column represents the mean±SD of at least triplicate experiments. **, $p<0.01$; and ***, $p<0.001$.

found no differences between freshly isolated Jurkat cells (Fig. 3A and B, grey shades) and A23187-treated ones (blue lines). In contrast, both indicators were present in cells treated with 10 μM of etoposide, a drug known to induce CD43 capping through caspase-activated apoptosis (red lines). To confirm that no caspases were involved, we pre-treated cells with the pan-caspase inhibitor Z-VAD-fmk, known to inhibit capping of CD43 in cells undergoing apoptosis (Fig. 3C, D and E) (Eda *et al.*, 2004). Z-VAD-fmk did not prevent capping of CD43 in A23187-treated Jurkat cells (Fig. 3C) nor did it prevent the recognition and the phagocytosis of these cells by macrophages (Fig. 3D and E), indicating that A23187-induced capping is independent from caspase activity. While A23187 may indeed cause apoptosis under higher concentrations and with protracted treatment (Kim *et al.*, 2002; McConkey *et al.*, 1989b, 1989a), results here indicate that calcium elevation alone induced capping of CD43 without inducing apoptosis.

AM group of calcein-AM is hydrolysed by intracellular esterases, yielding the membrane-impermeable green-fluorescent calcein. Apoptotic and dead cells with compromised cell membranes do not retain calcein. Calcein-AM is therefore used as a membrane-permeable live-cell labeling dye (Hirt *et al.*, 2000). A23187-treated Jurkat cells were engulfed with still intact plasma membrane, as they retained calcein during the phagocytosis procedure (Fig. 3F, upper panels and lower left panel). As shown in Fig. 3F lower right panel, the calcein-fluorescence was dispersed inside of macrophage, indicating that the phagocytosed cells were lysed by macrophages. Taken together, these observations suggest that A23187-treated cells were regarded as removal target for macrophages while still alive.

CD43 and its carbohydrate chains are the recognition ligands for A23817-treated Jurkat cells

We next investigated whether or not CD43 carbohydrate chains are the ligands for macrophage detection of A23187-treated cells. Macrophage recognition was inhibited in Jurkat cells pretreated with anti-CD43 antibody (Fig. 4A), indicating that CD43 is the ligand. Further, poly-*N*-acetyllactosaminyl carbohydrates containing oligosaccharides prepared from human erythrocyte membranes (Beppu *et al.*, 1996b) also inhibited the recognition (Fig. 4B), indicating that recognition likely occurs through the poly-*N*-acetyllactosaminyl chains on the CD43 caps.

Nucleolin is the macrophage receptor for A23187-treated Jurkat cells

CD43-capped apoptotic cells are known to be recognized by nucleolin (Hirano *et al.*, 2005; Miki *et al.*, 2009, 2007), and we confirmed that this was the case in A23187-treated CD43-capped Jurkat cells. First, macrophages were inhibited from binding to A23187-treated Jurkat cells by pre-treatment with anti-nucleolin antibody, while control rabbit IgG was ineffective (Fig. 5A). Additionally, we induced expression of recombinant nucleolin on the surface of non-macrophage HEK cells as described previously (Hirano *et al.*, 2005) and found that more A23187-treated Jurkat cells bound to these HEK cells than to non-transfected HEK cells (Fig. 5B), and that binding was inhibited by oligosaccharides prepared from human erythrocytes (Fig. 5C). These results clearly indicate that nucleolin is the receptor for A23187-treated Jurkat cells.

CD43-mediated recognition of ionomycin-treated Jurkat cells

Ionomycin is also used as a calcium ionophore to trigger calcium-increase in Jurkat cells (Nair *et al.*, 2006). We next tested whether or not elevated calcium with ionomycin induces CD43 capping and subsequent recognition by macrophages via the CD43-mediated mechanism. We observed

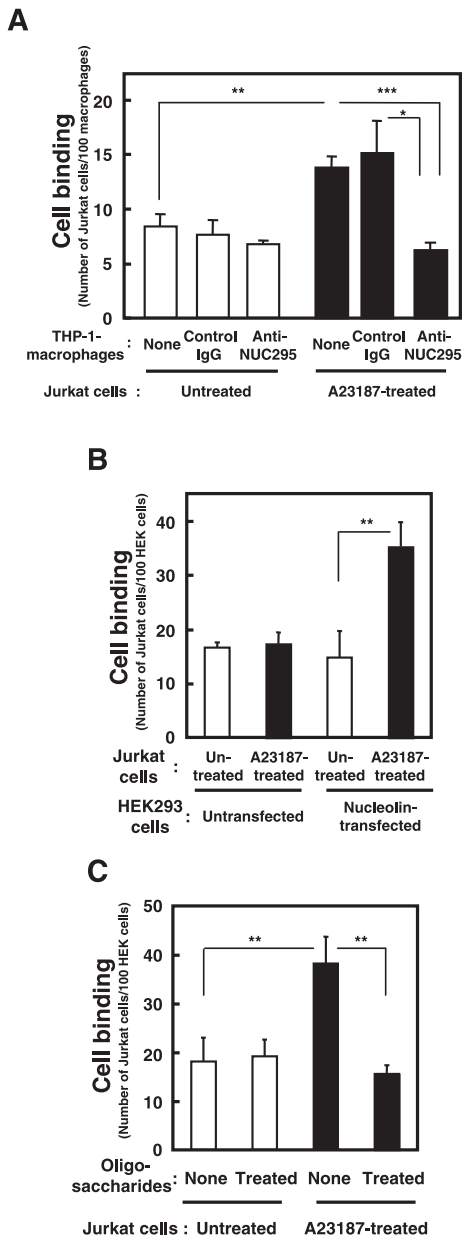

Fig. 5. Nucleolin is the macrophage receptor for A23187-treated Jurkat cells. (A) Anti-NUC295 antibody inhibits binding of macrophages to A23187-treated Jurkat cells. (B) Binding of A23187-treated Jurkat cells to nucleolin-transfected HEK cells. (C) Oligosaccharides from erythrocyte membranes inhibit binding of nucleolin-transfected HEK cells to A23187-treated Jurkat cells. Each bar represents the mean±SD of at least triplicate experiments. *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$.

that while CD43 distribution on the surface of Jurkat cells was uniform on Jurkat cells (Fig. 6A, upper panel), distribution was localized in a cap on ionomycin-treated Jurkat cells (Fig. 6A, lower panel). Capping required a minimum of 20 nM of ionomycin (Fig. 6B). Because ionomycin is known to cause apoptosis under certain conditions (Gil-Parrado *et al.*, 2002), we examined PS expression on 20 nM or 2 μM ionomycin-treated cells. We hardly found a difference between ionomycin-untreated Jurkat cells (Fig. 6C, grey shades) and 20 nM ionomycin-treated ones (blue line). In contrast, PS expression was increased in cells treated with 2 μM of ionomycin (red line). Indicating that 20 nM of ionomycin-treatment induced capping of CD43 without inducing apoptosis. In addition, ionomycin-treated Jurkat cells were recognized by macrophages, and the recognition was inhibited by pretreatment with anti-CD43 antibody (Fig. 6D). Imboden *et al.* reported that >5 nM of ionomycin-treatment increase intracellular calcium levels in Jurkat cells (Imboden *et al.*, 1985). Additionally, BAPTA-AM, an intracellular calcium chelator, -treatment inhibited the recognition (Fig. 6E). These results indicating that ionomycin-treated cells with elevated calcium were also recognized by macrophages via the CD43-mediated mechanism.

Discussion

Here we demonstrated that Jurkat cells with artificially raised calcium levels were recognized and phagocytosed by macrophages through the interaction between the cell-surface poly-*N*-acetyllactosaminyl carbohydrate chains of CD43 and the macrophage receptor nucleolin. Further, these cells were removed while still alive, indicating that calcium elevation alone can cause a cell to be regarded by macrophages as a target for removal. Although many details regarding the mechanism by which macrophages recognize apoptotic cells are known, how they recognize live-but-deteriorating cells is poorly understood. This study sheds light on the latter process and highlights the important role of CD43 capping with poly-*N*-acetyllactosaminyl carbohydrate chains. These findings contribute to the broader understanding of biological defense mechanisms.

We used A23187 to induce elevated calcium levels in our Jurkat cells. Indeed, A23187 are often used in the laboratory to increase intracellular calcium (Sasaki and Hasegawa-Sasaki., 1985; Minetti *et al.*, 1996). Additionally, when treated with Ca^{2+} and A23187, human erythrocytes and lymphocytes respond by budding to release membrane vesicles (Allan and Michell, 1975; Allan *et al.*, 1976a; Podszywalow-Bartnicka *et al.*, 2007). This treatment also induces several other biochemical changes, including K^+ efflux (Lew and Ferreira, 1976; Dupuis *et al.*, 1989), the breakdown of polyphosphoinositides, a rise in 1,2-diacylglycerol and phosphatidate concentrations (Allan *et al.*, 1976b; Allan and Michell, 1978; Ponnappa *et al.*, 1980; Allan and Thomas, 1981; Butikofer *et al.*, 1993), and a selective loss of glycosylphosphatidylinositol-anchored membrane proteins (Butikofer *et al.*, 1993). The treatment of hematopoietic cells with A23187 can thus be regarded as a model with which to investigate the biochemical mechanisms responsible for membrane composition during intracellular calcium elevation.

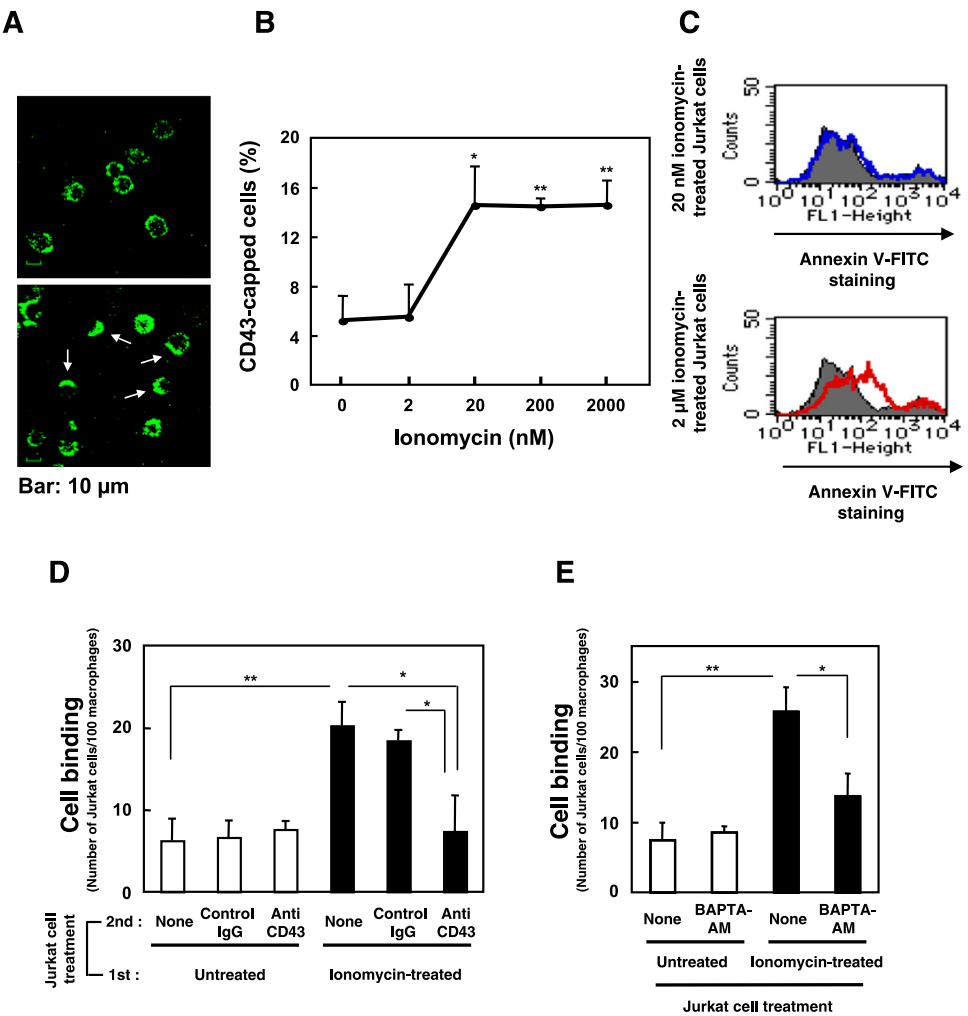

Fig. 6. CD43-mediated recognition of ionomycin-treated Jurkat cells. (A) Distribution of CD43 on Jurkat cells was observed using confocal immunofluorescence microscopy (FV1000D). CD43 distribution on untreated Jurkat cells (upper panel) and cells treated with 20 nM of ionomycin for 1 h (lower panel). The arrows indicate capping of CD43. (B) Changes in CD43 capping on Jurkat cells as a function of concentration of ionomycin-treatment. (C) Measurement of PS expression. Jurkat cells were treated with 20 nM ionomycin (upper panel, blue line) or 2 μM ionomycin (lower panel, red line) at 37°C for 1 h. Grey shades, fresh Jurkat cells. (D) Anti-CD43 antibody inhibits binding of macrophages to 20 nM ionomycin-treated Jurkat cells. (E) Effect of co-incubation with 10 μM intracellular calcium chelator BAPTA-AM on the recognition of 20 nM ionomycin-treated Jurkat cells. Each column represents the mean±SD of at least triplicate experiments. Statistical analysis was conducted between the control and indicated sample. *, $p<0.05$; and **, $p<0.01$.

Although we showed that cells with elevated calcium levels developed CD43 caps on the cell surface, currently we are unable to explain the capping mechanism definitively. However, studies have shown that increased intracellular calcium induces CD32 and CD44 to form patches or caps (Roberts *et al.*, 1997; Bourguignon *et al.*, 1993). Authors speculated that CD44 formed caps through the activity of the calcium ionophore ionomycin as follows: Increased calcium binds to calmodulin, Ca/calmodulin induces phosphorylation of myosin L chains, the membrane-associated actomyosin contractile-system is engaged, and CD44 forms caps (Bourguignon *et al.*, 1993). Cytoskeletal proteins may therefore play a critical role in ligand capping in cells with elevated calcium. Our previous research also suggests that active movement of cytoskeleton proteins may induce CD43 capping when caspases are activated (Eda *et al.*, 2004; Oguri *et al.*, 2012). Activated caspases can abrogate the link between CD43 and actin during the early stages of apoptosis (Kondo *et al.*, 1997), and disrupting the actin bond presumably causes active movement of other cytoskeleton proteins that then induce capping of CD43 (Eda *et al.*, 2004; Oguri *et al.*, 2012). However, while the capping of CD43 on etoposide-treated cells is inhibited by Z-VAD-fmk, this is not the case with A23187-treated cells, indicating that the bond can be disrupted by other means (Oertner and Matus, 2005; Bourguignon and Pressman, 1983). Although acti-

vated caspases sever the link between CD43 and actin, calcium influx may also disturb this important connection. Alternatively, since increased calcium is known to induce active movement of cytoskeleton proteins that can lead a capping (Oertner and Matus, 2005; Bourguignon and Pressman, 1983), even if the CD43-actin bond is not severed, stronger active movement of other cytoskeleton proteins triggered by excessive calcium influx may induce CD43 capping. Thus, disrupting the balance of tension between CD43 and cytoskeleton proteins is thought to be a key factor in cap formation. Further investigation is needed to illuminate the precise details of the capping mechanism.

Capping or clustering of CD43 is found in interactions between neutrophil cells and vasclcular endothelial cells that are treated with chemotaxis factors such as *N*-formyl-methionyl-leucyl-phenylalanine, *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine, and IL-8, resulted in neutrophil-chemotax to outside of blood vessel (Alonso-Lebrero *et al.*, 2000; Seveau *et al.*, 2001; Dehghani Zadeh *et al.*, 2003). These findings supporting the idea that capping of CD43 induce cell-cell interactions. However, how the capping of CD43 causes various physiologic activities is not fully understood P-selectin glycoprotein ligand-1 and intracellular adhesion molecule-3 exist in the area of capped CD43 of chemotactic neutrophil (Alonso-Lebrero *et al.*, 2000; Seveau *et al.*, 2001; Dehghani Zadeh *et al.*, 2003). The composition of the molecules that work together in CD43 cap formation may influence the physiologic activities.

The mechanism by which macrophages recognize cells with elevated calcium is poorly understood. Using A23187-treated human erythrocytes and lymphocytes, Balasubramanian *et al.* revealed that sustained elevation in cytosolic Ca^{2+} leads to outward movement of plasma-membrane PS (Balasubramanian *et al.*, 2007), which has since been presumed to cause the proteolytic degradation of cytoskeletal proteins seen after increases in intracellular calcium (Verhallen *et al.*, 1987, 1988; Bevers *et al.*, 1983). Because PS is already a well-known ligand for macrophage recognition (Savill *et al.*, 1993; Savill and Fadok, 2000; Fadok *et al.*, 2001; Platt *et al.*, 1998; Eda *et al.*, 2004), these previous findings suggest it to be the relevant ligand for the recognition of cells with elevated calcium levels.

Understanding how cells with elevated calcium are removed is important because increased intracellular calcium is a symptom of several pathologies, including Alzheimer's disease and SLE. These diseases may be ameliorated or prevented by the removal of harmful cells (Feske, 2007; Liossis *et al.*, 1996; Saido *et al.*, 1994; Saito *et al.*, 1993; Tsuji *et al.*, 1998; Lee *et al.*, 1991; Hajimohammadreza *et al.*, 1997; Saatman *et al.*, 1996; Nakagawa *et al.*, 2000). Indeed, amyloid β induces calcium influx into neurons which in turn can cause neurotoxicity (apoptosis) that results in Alzheimer's disease (Ho *et al.*, 2001). Milk fat globule epidermal growth-factor 8 (MFG-E8), which initially attaches to PS on apoptotic cells and then forms a link with phagocytes to initiate or augment phagocytosis (Hanayama *et al.*, 2002), may prevent Alzheimer's disease in the brain via microglia that aid in clearing amyloid β-induced apoptotic neurons (Fuller and Van Eldik, 2008). Additionally, PS-MFG-E8-mediated clearance also prevents SLE (Hanayama *et al.*, 2004). Just as both the PS and capped-CD43 pathways lead to successful removal of deteriorating (apoptotic) cells (Yamanaka *et al.*, 2005), we propose that in addition to PS-mediated removal, Alzheimer's disease and SLE may also be prevented by carbohydrate-mediated removal of cells with elevated calcium in the early stage before cells die.

Nucleolin was confirmed as the receptor for the CD43 caps on calcium-elevated cells. Nucleolin is present in the nucleus, cytoplasm, and on the surface of some types of cells including macrophages (Ginisty *et al.*, 1999; Nigg, 1997). Previous studies have shown that macrophages utilize nucleolin to recognize and phagocytose apoptotic and oxidized cells (Hirano *et al.*, 2005; Miki *et al.*, 2009) as well as living cells that have been treated with cytochalasin B (Oguri *et al.*, 2012). Nucleolin-mediated recognition by macrophages is therefore simple and powerful, as indicated by the indiscriminant removal of capped cells. Nucleolin is also a receptor for lipoproteins (Semenkovich *et al.*, 1990), coxsackie B virus (de Verdugo *et al.*, 1995), human immunodeficiency virus (Nisole *et al.*, 2002), human parainfluenza virus type 3 (Bose *et al.*, 2004), and enterohemorrhagic *Escherichia coli* O157:H7 (Sinclair and O'Brien, 2002). Further, nucleolin can bind to anionic molecules such as DNA, RNA (Jordan, 1987; Nigg, 1997), and, as confirmed here, poly-*N*-acetyllactosaminyl chains. Taken together, these observations suggest that nucleolin on the surface of macrophages may have a general scavenger receptor-like ability.

Several studies have reported that nucleolin forms spot-like aggregations on cell surfaces (Said *et al.*, 2002; Sinclair and O'Brien, 2002). We also observed this phenomenon on nucleolin-transfected HEK cells (data not shown). The three-dimensional structure of the CD43 cap may therefore be critical for multivalent binding with aggregated nucleolin and might also explain why macrophages cannot recognize CD43 if the protein is uniformly distributed (Oguri *et al.*, 2012).

Here we revealed that increased intracellular calcium induces capping of CD43 and removal by macrophages. Increases in intracellular calcium occur in diverse pathologies including SLE, cancer, common variable immunodeficiency glutamate, kainite, maitotoxin, brain trauma, and neurodegenerative processes such as Alzheimer's disease (Feske, 2007; Liossis *et al.*, 1996; Saido *et al.*, 1994; Saito *et al.*, 1993; Tsuji *et al.*, 1998; Lee *et al.*, 1991; Hajimohammadreza *et al.*, 1997; Saatman *et al.*, 1996; Nakagawa *et al.*, 2000). Although these are critical diseases, more details are required to understand them fully. If calcium-elevation causes these diseases, disruption of our clearance-mechanism may be involved in the development of these diseases.

Therefore, the mechanism may be performed as physiological defense mechanism against calcium-elevation. In contrast, the mechanism may be involved in the aggravation of these diseases and complication with hypercalcemia, such as renal insufficiency, by removal of cells (Ossareh, 2011). Our present findings may contribute to the broader understanding of developmental mechanisms and thus medical treatment or prevention of these diseases. Further study for the removal mechanism of calcium-elevated cells is necessary for better understanding of the disease with calcium-elevation and tissue homeostasis.

Conflicts of interest

The authors report no conflicts of interest.

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