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Purinergic receptor agonists modulate phagocytosis and clearance of apoptotic cells in macrophages

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ABSTRACT

Phagocytosis plays an important role in controlling inflammation and antigen cross-presentation through the uptake of apoptotic bodies from dying cells. As dying cells are known to release nucleotides and other "danger signals", we investigated whether extracellular nucleotides may affect phagocytosis through binding to P2 purinergic receptors on phagocytic cells. We here show that the purinergic receptor agonists, ATP, ADP, α,β-methylene ATP (α,β-meATP), 3′-O-(4-benzoyl)benzoyl ATP, UTP and UDP, increased phagocytosis of latex beads, and some of them increased endocytosis and/or macropinocytosis of dextran by macrophages. The enhanced phagocytosis could be inhibited by pre-treatment with the P2X and P2Y antagonists, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid and suramin, and the $P2Y_1$-selective antagonist, MRS2179. The nucleotides induced upregulation in macrophages of the β2 integrin CD11b/CD18 (Mac-1) and the vitronectin receptor ($\alpha_v\beta3$, CD51/CD61), both of which are involved in recognition and internalization of apoptotic cells. In addition, ATP and α,β-meATP increased adhesion of apoptotic cells to macrophages, both *in vitro* and *in vivo*, and α,β-meATP had a small effect on adhesion of necrotic cells. The nucleotides had no effect on adhesion of viable cells. We propose that engagement of the P2 receptors ($P2X_1$, or $P2X_3$) by extracellular nucleotides released from dying cells increases the ability of macrophages to bind apoptotic bodies, thus enhancing their ability to internalize and present antigens from the dying cells.

Introduction

Macrophages interact with the adaptive immune system by means of two main signals: (i) recognition of MHC–peptide complexes expressed on the surface of macrophages by antigen-specific T cells, and (ii) binding of co-stimulatory molecules, such as CD80/B7.1 and CD86/B7.2, on macrophages by CD28 molecules on T cells (Sharpe and Freeman 2002). Macrophages are also directly involved in maintaining homeostasis through clearance of apoptotic cells, in a manner that usually avoids stimulating an inflammatory response (Kono and Rock 2008). Discrimination between living, dying and dead cells is an essential requirement for appropriate clearance of apoptotic cells. Failure to clear dying cells may sometimes reflect an imbalance between the number of dead cells and the local availability of functional scavenger phagocytes. Factors that increase apoptosis (such as viral infections, local inflammation, and neonatal tissue remodeling) and factors that decrease the phagocytic ability of macrophages (such as phagocytic exhaustion and the cytokine milieu) may contribute to the accumulation of uncleared apoptotic bodies and breaking of immune tolerance (Maderna and Godson 2003).

Recognition of appropriate target cells may also be complicated by the fact that many of the ligands used for recognition of apoptotic cells, such as phosphatidylserine (PS) or calreticulin, are also expressed on viable cells, particularly following activation of the cells. One possibility to account for the discrimination between apoptotic and viable cells is that apoptotic cell recognition and subsequent engulfment require not only the exposure of "eat-me" signals but also redistribution of receptors into patches. Many of the ligand interactions are likely to be of low affinity, requiring oligomerization (an increase in avidity) for optimal stimulation. This could lead to the generation of a recognition "synapse" between the apoptotic cell surface and the phagocyte. A large reper-

Abbreviations: α,β-meATP, α,β-methylene ATP; BzATP, 3′-O-(4-benzoyl)benzoyl ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid.

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toire of phagocytic receptors is involved in apoptotic uptake, and includes receptors as diverse as CD11C, C1q, collectins, complement and CD14 (Devitt et al. 1998; Mevorach et al. 1998; Ogden et al. 2001; Taylor et al. 2005). Some of these receptors act through specific recognition of apoptotic ligands, enhancement of binding of apoptotic cells, and ability to phagocytose the dying cells.

Apoptosis is a physiological program of cell suicide that directs engulfment and safe destruction of cell corpses by healthy neighbouring cells or professional phagocytic scavengers such as macrophages (Fadok and Chimini 2001). The processes of apoptosis and phagocytosis work in concert to perform central roles in biological functions such as embryogenesis; mature tissue homeostasis; elimination of infected, aged, and injured cells; cellular immunity; and resolution of inflammation (Cohen 1993; Han et al. 1993; Hopkinson-Woolley et al. 1994). Uptake of apoptotic cells has been suggested to involve two separate steps. Individual or multiple engagement of several receptors including CD14, CD68, CD36 and $\alpha_v\beta3$ integrin results in binding of the particles (Henson et al. 2001). Adhesion ligands lead to attachment of the apoptotic cell (tethering), but they are not able to trigger internalization. Engagement of signaling receptors then leads to initiation of uptake.

The lack of an inflammatory response during apoptosis has been attributed to the rapid clearance of apoptotic cells before cell lysis, thereby preventing the release of noxious contents which could provoke inflammation and tissue damage. In fact, conditions causing apoptotic cell death are usually associated with suppression of inflammation and cell-mediated immunity (Kang et al. 1994; Meunier et al. 1995).

Phagocytes display a high level of redundancy in the receptors used for recognizing apoptotic cells, and are able to use several receptors simultaneously, such as the scavenger receptor CD36 and the β2 integrin CD11b/CD18 (Mac-1) (Savill et al. 2002). Clearance of apoptotic neutrophils and eosinophils is mediated in part by macrophages (Savill et al. 1990) and fibroblasts (Hall et al. 1994) via the vitronectin receptor $\alpha_v\beta3$ (CD51/CD61). The integrin family members $\alpha_v\beta3$ and $\alpha_v\beta5$ are thought to interact directly with C3b/bi binding sites on the apoptotic cell (Mevorach et al. 1998).

The extracellular nucleotides ATP and UTP are increasingly viewed as a new class of innate immune system mediators, which play a role in inflammation following their release at sites of inflammation as a result of cell damage. ATP is thought to mediate most of its effects through binding to the P2 purinergic receptor, $P2X_7$, while UTP can have its effects through $P2Y_2$ and $P2Y_4$ receptors (Bours et al. 2006). $P2X_7$ receptors in the immune system also mediate effects distinct from inflammation, including cell death, cell fusion and proliferation, and bone formation (Adinolfi et al. 2002; Falzoni et al. 2000; Ke et al. 2003; Lemaire et al. 2006). With regards to cell death and inflammation, stimulation of the $P2X_7$ receptor results in 'flipping' of PS from the inner leaflet to the outer leaflet, so that it is exposed at the cell surface of lymphocytes, and this altered lipid distribution alters the activity of at least three distinct membrane proteins (Elliott et al. 2005). P2X and P2Y receptors are involved in the activation of dendritic cells, which is necessary for efficient initiation of immune responses (Coutinho-Silva et al. 1999; Gallucci and Matzinger 2001; Mutini et al. 1999). In fact, besides antigen, dendritic cells require a second signal in order to activate specific T lymphocytes, and necrotic cells could provide the signal via extracellular ATP. Exposure of dendritic cells to necrotic, but not apoptotic cells, results in dendritic cell maturation (Sauter et al. 2000), and phagocytosis by dendritic cells of apoptotic cells mixed with necrotic cells results in higher expression of proinflammatory cytokines and upregulated expression of CD11b (Chen et al. 2001), which along with the vitronectin receptor, CD51/61 ($\alpha_v\beta3$), is involved in clearance of apoptotic bodies and cells (Freire-de-Lima et al. 2000; Mevorach et al. 1998; Monks et al. 2005; Savill et al. 1990).

The ability of professional phagocytes to clear apoptotic cells in a non-inflammatory manner relies on the expression by the dying cells of eat-me signals and other specific ligands of dying cells. The expression of these apoptotic signals is common to all physiological forms of cell death, regardless of the stimulus that triggered cell death (Cocco and Ucker 2001; Cvetanovic and Ucker 2004). Cells that die by necrosis are also recognized by professional phagocytes; however, necrotic corpses do not downregulate inflammatory responses and, instead, usually promote inflammation.

It is not clear how macrophages recognize necrotic cells (Krysko et al. 2006). While it was suggested that some macrophage receptors involved with engulfment of apoptotic cells also contribute to the uptake of necrotic cells (Bottcher et al. 2006), recognition of apoptotic and necrotic cells may take place through distinct mechanisms (Cocco and Ucker 2001). Recent studies indicate that the interaction of macrophages with dying cells initiates internalization of the apoptotic or necrotic targets, and that internalization can be preceded by "zipper"-like and macropinocytotic mechanisms, respectively (Krysko et al. 2006).

In general, the class of P2 receptors displays a wide range of phagocytic and chemotactic properties (Bours et al. 2006); and recently UDP, via $P2Y_6$ activation, has been implicated in phagocytosis of apoptotic bodies in microglial cells (Koizumi et al. 2007). The receptors $P2X_{1-7}$ are expressed in macrophages where functional $P2X_1$, $P2X_4$ and $P2X_7$ receptors were observed (Coutinho-Silva et al. 2005; Sim et al. 2007), but their function in immune responses remain largely uncharacterized.

We here consider the attachment mechanisms involved in phagocytosis of apoptotic cells as well as the contribution that P2X receptors may make. We also discuss the potential of impaired P2X-dependent phagocytosis in the pathogenesis of inflammation.

Materials and methods

Reagents

ATP, α,β-meATP, ADP, apyrase, 3′-O-(4-benzoyl)benzoyl ATP (BzATP), pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), brilliant blue green (BBG), suramin, oxidized ATP (oATP), UTP, UDP, MRS2179, 2 μm FITC-conjugated latex beads and FITC-dextran (40 kDa) were purchased from Sigma (St. Louis, MO). (1-[2-(5-Carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methyl-phenoxy)-ethane-N,N,N′,N′-tetraacetic acid) (FURA-2) and cytochalasin were from Calbiochem. Anti-$P2X_{1-3}$ antibodies were from Roche Bioscience (Palo Alto, CA), while FITC-conjugated rat anti-mouse CD11b/Mac-1 antibody and FITC- and PE-conjugated rat anti-mouse IgG2b isotype control (0.5 μl/10^6 cells) were from PharMingen (San Diego, CA). PE-conjugated anti-CD51 antibodies (10 μl/10^6 cells) and FITC-conjugated anti-CD61 antibodies (10 μl/10^6 cells) were from Serotec (Kedlington, Oxford, UK).

Animals

Breeding, maintenance and killing of the animals used in this study followed the principles of good laboratory animal care and experimentation in compliance with the Carlos Chagas Filho ethical regulations, and the protocols were approved by the Animal Care and Use Committee (IBCCF 039). Animals were kept at a constant 12 h/12 h light–dark cycle with free access to food and water. This study was carried out with adult male Swiss Webster mice weighing between 20 and 25 g. C57Bl/6 mice were purchased from BioRio Foundation (Rio de Janeiro, Brazil). $P2X_7R$ knock-out ($P2X_7R^{-/-}$)

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mice derived from Pfizer (Groton, CT, USA) were supplied by Dr. James Mobley (PGRD, Pfizer Inc., Groton, USA) and bred at the Transgenic Mice Laboratory at the Biophysics Institute Carlos Chagas Filho, Federal University of Rio de Janeiro. Animals were killed by exposure to an increasing dose of carbon dioxide and death was confirmed by cervical dislocation.

Macrophages

Peritoneal macrophages were obtained from a peritoneal cavity wash with phosphate-buffered saline (PBS) containing 4 units apyrase/ml. Cell viability following peritoneal wash was over 95% in all cases, as measured by Trypan blue exclusion. The enriched

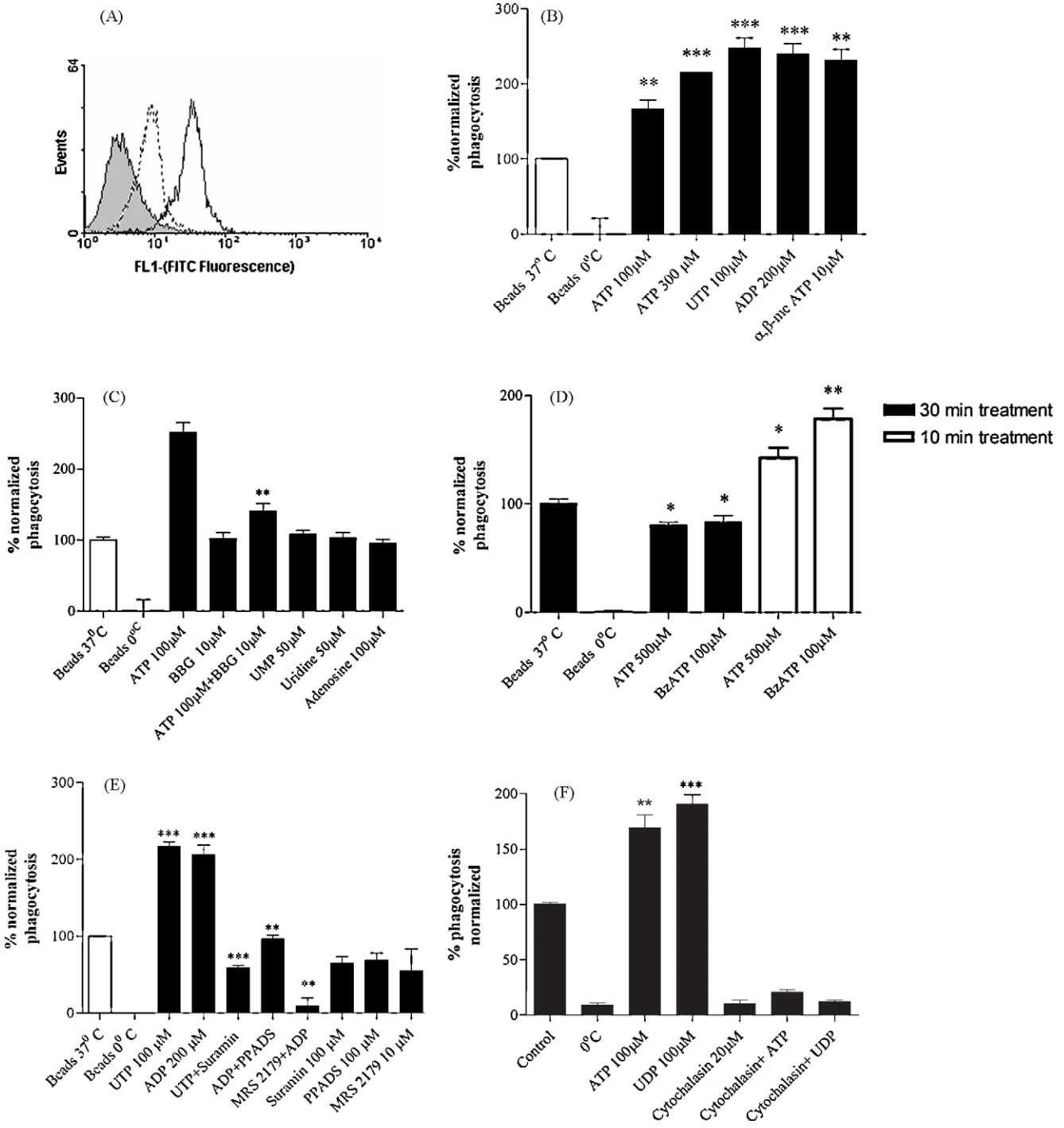

Fig. 1. Phagocytosis of latex beads is modulated by extracellular nucleotides. Peritoneal macrophages were plated for 24 h, treated with nucleotides for 30 min, and then incubated with latex beads. (A) A typical flow cytometry profile of cells treated with ATP and exposed to FITC-conjugated latex beads. The grey histogram represents phagocytosis at 0 °C, the dotted line shows control phagocytosis at 37 °C without nucleotides, and the dark line corresponds to phagocytosis of latex beads in cells treated with ATP. (B) Cells were treated with α,β-meATP, ATP, UTP or ADP, and exposed to FITC-conjugated latex beads. (C) Cells were pre-treated 1 h with brilliant blue green (BBG) followed by treatment with ATP; or treated with UMP, uridine or adenosine; or (E) pre-treated for 1 h with suramin, MRS 2179 or PPADS, then treated with UTP or ADP. (D) Cells were treated with ATP or BzATP for 30 or 10 min; or (F) pre-treated for 30 min with cytochalasin, then treated with ATP or UDP and exposed to FITC-conjugated latex beads for 30 min. The number of fluorescent latex beads taken up per macrophage is represented as mean fluorescence intensity (MFI) of FITC-positive macrophages measured by flow cytometry. The data were normalized to phagocytosis obtained with untreated macrophages $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls. Bar, 20 µm.

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mononuclear cells were adjusted to 2×10^6 cells/ml and transferred to Dulbecco's Modified Eagle Medium (DMEM; from Gibco BRL, Paisley, Scotland) containing 5% heat-inactivated FCS, 2 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 units apyrase/ml, and cells were plated on glass coverslips or 96-multiwell plates. After one hour of incubation at 37 °C in a 5% CO_2 humidified atmosphere, non-adherent cells were removed by vigorous washing and the adherent cells (approximately 60% of the original cell suspension) were kept in the same conditions for 24–48 h until use. The mouse macrophage cell line J774.G8, derived from the original J774.A1 from the American Type Culture Collection (ATCC, Rockville, MD), was also used. This cell line was grown in complete DMEM, supplemented as described above, on 25 cm^2 plastic culture flasks (Corning, NY, USA) at 37 °C in a 5% CO_2 humidified atmosphere.

Endocytosis assay

Peritoneal macrophages were placed in sterile 96-well plates and incubated with different concentrations of ATP, ADP, UTP, UDP, BzATP and α,β-meATP for 30 min, then incubated with 0.5 mg/ml 40 kDa FITC–dextran (Sigma) in DMEM culture medium without serum for 30 min at 37 °C in a humidified atmosphere with 5% CO_2 or 0 °C as negative control. Cells were washed until all particles that had not been endocytosed were removed. Fluorescence was measured using a microplate fluorescence reader (BIO-TEK Instruments, Inc.; VT) at an excitation wavelength of 485 nm and emission of 528 nm. Alternatively J774 cells were plated in 12-well dishes for 48 h, after which the cells were incubated with or without nucleotides. Endocytosis activity was assessed by simultaneous addition of 0.5 mg/ml 40 kDa FITC–dextran in RPMI without serum for 30 min at 37 °C (controls were incubated on ice). Cells were harvested in cold PBS and the number of FITC–dextran particles taken up per macrophage was represented by mean fluorescence intensity (MFI) of FITC-positive macrophages measured on flow cytometry. The data were normalized to endocytosis obtained on untreated macrophages (MFI of untreated macrophages at 37 °C).

Phagocytosis assay

Macrophages were placed in sterile 96-well plates, pre-incubated for 1 h with oATP, suramin, PPADS, MRS2179, cytochalasin or control buffer, then treated with different concentrations of ATP, UTP, UDP, BzATP, ADP and α,β-meATP for 30 min, followed by incubation with 10:1 (beads:cells) FITC-conjugated latex beads in culture medium for 30 min at 37 °C in a humidified atmosphere with 5% CO_2 or 0 °C as negative control. Cells were washed until all particles that had not been phagocytosed were removed. The number of fluorescent latex beads taken up per macrophage was represented by mean fluorescence intensity (MFI) of FITC-positive macrophages measured by flow cytometry. The data were normalized to phagocytosis obtained on untreated macrophages (MFI of untreated macrophages at 37 °C).

Apoptotic and necrotic cells

Lymphocytes obtained by peritoneal wash were suspended in complete medium and heated (56.8 °C for 30 min) to obtain apoptotic cells, or subjected to four cycles of freezing (−70 °C) and thawing (37 °C) to obtain necrotic cells as described previously (Freire-de-Lima et al. 2000; Sauter et al. 2000). The procedure for induction of apoptosis in lymphocytes was confirmed by a high level of annexin V staining and low permeability to propidium dyes, showing that 90% of lymphocytes were apoptotic after treatment. Apoptotic (Apo-1), necrotic (Nec-1) or viable cells (lymphocytes)

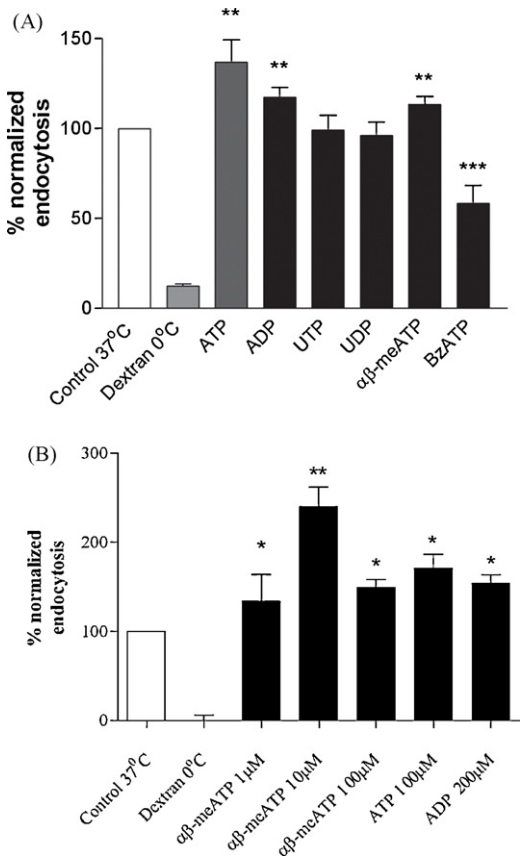

Fig. 2. Dextran endocytosis is enhanced by nucleotides. The J774 cell line (A) and peritoneal macrophages (B) were plated for 24 h, treated with 200 μM nucleotides (A) for 30 min and then exposed to FITC–dextran for 30 min (control on ice). Cells were analyzed by (A) flow cytometry and (B) microplate fluorescence reader, and endocytosis values (relative fluorescence) were normalized with respect to values for FITC–dextran alone at 37 °C, as described in "Materials and methods" section. Values represent the average and SEM of 3 separate experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls.

(10^6 cells per well) were added to macrophages (10 apoptotic cells:1 macrophage) after several washings.

In vitro adhesion assay

Macrophages were treated with nucleotides or control buffer for 30 min, then cultured with Apo-1 or Nec-1 cells for 3 h, washed gently in order to release cells that had not adhered, fixed with 4% paraformaldehyde, and counted under phase-contrast microscopy. Adhesion was calculated as the percentage of rosetted macrophages out of a total of 100 macrophages counted per well. In some experiments, the cells were exposed to Apo-1-FITC for 3 h, and then washed until all apoptotic bodies that had not been phagocytosed were removed. The cells were fixed with paraformaldehyde, stained with Evans blue (which stains the cytoplasm in red) and DAPI (staining nuclei in blue). The microscopy imaging of phagocytosis of apoptotic cells was performed with an LSM 510 META confocal microscope (Zeiss, Germany) or a Zeiss Axiovert 200 M microscope equipped with an ApoTome slide and fluorescence optics (Zeiss, GmbH, Germany).

In vivo adhesion assay

We adapted the method described previously (Potter et al. 2003), using a "peritoneal test tube" model. Briefly, mice received an intraperitoneal injection of 1 ml of 500 μM ATP or control buffer.

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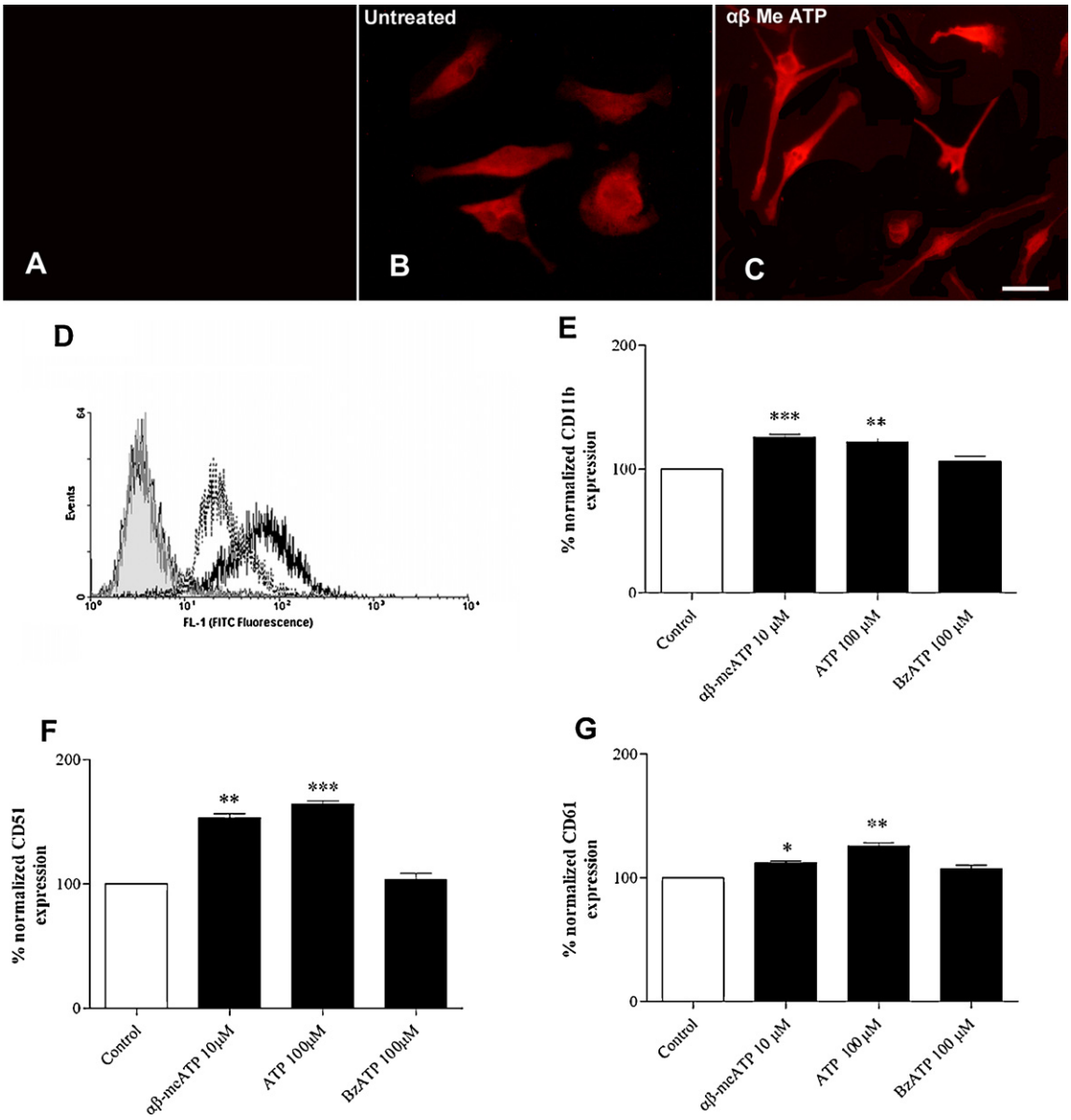

Fig. 3. Expression of adhesion molecules is increased by nucleotides. Peritoneal macrophages were plated for 24 h, treated first with α,β-meATP, ATP or BzATP at the indicated doses and then incubated for another 30 min in a CO_2 incubator. The reaction was stopped by washing with cold buffer. Cells were incubated with FITC-conjugated (A–E) anti-CD11b, (F) PE-conjugated anti-CD51, or (G) FITC-conjugated anti-CD61 antibodies on ice for an additional 30 min and then analyzed by (D–G) flow cytometry (mean intensity fluorescence shown in panels E–G) or (A–C) immunofluorescence microscopy. The immunofluorescence micrographs show (A) negative controls performed by omitting the primary Ab and incubating with secondary antibody, (B) control cells without nucleotide treatment, and (C) cells treated with α,β-meATP. In the fluorescence plot in (D), the grey histogram represents isotype control, the dotted line shows CD11b staining without nucleotides, and the dark line corresponds to CD11b staining in cells treated with α,β-meATP. Values represent the average and SEM of 3–5 separate experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

After 30 min, 10^7 murine lymphocytes (in 1 ml) that had undergone apoptosis (Apo-1) were inoculated intraperitoneally. After 3 h, peritoneal cells were recovered by lavage with ice-cold buffer, and adhesion was assessed by direct counting. In some experiments using P2X antagonists, 500 μM of PPADS or TNP-ATP were injected 1 h prior to ATP treatment.

Immunostaining

Peritoneal macrophages were obtained as described and plated on 8-well LAB-TEC slides (Nalge Nunc Inter, Naperville, UK) or 12-well plates (Corning USA) for 24–48 h before immunostaining. The cells were post-fixed for 10 min at room temperature in 4% paraformaldehyde prepared in PBS. Blocking of non-specific binding sites was achieved by pre-incubation with normal horse serum (NHS; Harlan Sera-Lab, Belton, UK) in PBS containing 0.05% merthiolate (Sigma) at room temperature for 20 min, before incubating with FITC-conjugated rat anti-mouse CD11b (1:1000) overnight at 4 °C. Cells were washed three times with PBS and mounted. Images were acquired with a Zeiss Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany) using Image Pro Plus software (Olympus).

Flow cytometric analysis

J774 and peritoneal macrophages were placed in sterile 24-well plates for 24 h and incubated with different concentrations of ATP, ADP and α,β-meATP for 30 min. The cells were then detached using EDTA solution (1 mM EDTA in PBS). Cells were centrifuged and resuspended in 4% paraformaldehyde for 20 min at room temperature. Cells were then washed twice in PBS, followed by blocking

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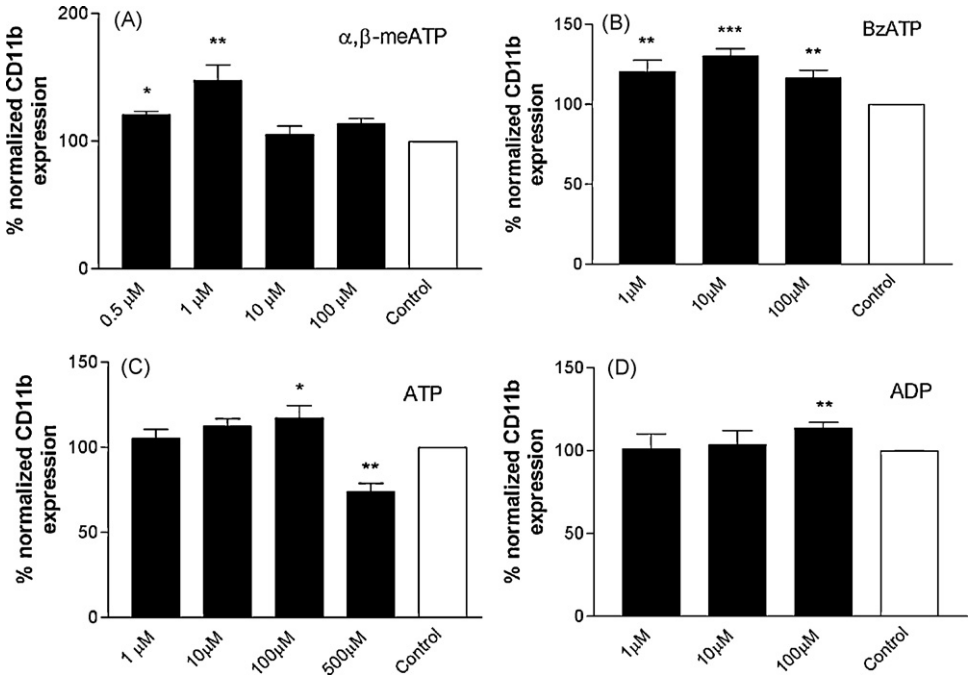

Fig. 4. Expression of the adhesion molecule CD11b is increased by nucleotides. J774 macrophages were plated for 24 h, treated first with (A) α,β-meATP, (B) BzATP, (C) ATP or (D) ADP at the indicated doses and then incubated for another 30 min in a CO_2 incubator. The reaction was stopped by washing with cold buffer. Cells were incubated with FITC-conjugated anti-CD11b antibodies on ice for an additional 30 min and then analyzed by flow cytometry. Mean intensity fluorescence values are shown in the histograms. Values represent the average and SEM of 3–5 separate experiments performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

of non-specific binding sites with NHS or anti-CD16/32 (clone 2.4G2) in PBS containing 0.05% merthiolate at room temperature for 1 h. Incubation with 1:100 FITC-conjugated rat anti-mouse CD11b, PE-conjugated CD51, FITC-conjugated CD61 antibodies or FITC-conjugated and PE-conjugated IgG2b isotype controls for 1 h was followed by gentle washing with PBS twice. The samples were transferred into 12 mm × 75 mm FALCON 2052 FACS tubes (Becton Dickinson, San Jose, CA), and data from 10,000 cells were collected on a FACScan flow cytometer (Becton Dickinson) with an argon laser tuned to 488 nm and analyzed using Cell Quest 3.3. The events acquired for analysis were gated to eliminate cell aggregates using WinMDI software (Multiple Document Interface Flow Cytometry Application 2.8).

Statistical analysis

The data shown represent the means ± SEM ($n = 3–5$). Statistical significance was estimated with the Student's *t*-test for unpaired observations or one-way ANOVA followed by Dunnett's multiple comparison when more than two means were considered. A value of $P < 0.05$ was considered significant.

Results

Nucleotides enhance the extent of phagocytosis

Pre-incubation of peritoneal macrophages with nucleotides for 30 min resulted in an increase of the phagocytic activity. As shown in Fig. 1A, latex beads were phagocytosed at higher levels when exposed to ATP (dark line), compared to control at 37 °C (dotted line). The P2 agonists ATP, ADP, UTP, UDP and α,β-meATP increased the ability of macrophages to phagocytose latex beads by 67 ± 12%, 140 ± 13%, 148 ± 13%, 91 ± 8% and 131 ± 15%, respectively; while UMP, uridine and adenosine had no effect (Fig. 1B–D, F). Conversely, a 30 min treatment with the $P2X_7$ receptor agonists, BzATP (100 μM) or ATP (500 μM), reduced the ability of macrophages to phatocytose latex beads by 17 ± 6% and 20 ± 2%, respectively. However, when macrophages were exposed briefly (10 min) to high concentrations of ATP (500 μM) or BzATP (100 μM), phagocytosis by the macrophages increased again by 42 ± 10% and 78 ± 11%, respectively (Fig. 1D). The $P2X_7$ antagonists BBG or oATP (not shown) partially antagonized the stimulatory effect of ATP (Fig. 1C); while the non-selective antagonists, suramin (for P2 receptors) and PPADS (for P2X receptors), and the $P2Y_1$ antagonist, MRS2179, also abolished the responses to UTP and ADP (Fig. 1E). The inhibitor of actin-dependent phagocytosis, cytochalasin, also inhibited phagocytosis induced by ATP and UDP (Fig. 1F). ATP-induced phagocytosis was partially inhibited by pre-exposure of the cells to the calcium chelator EGTA (46 ± 7%, $P < 0.001$), while UDP-induced phagocytosis was not affected (12 ± 11%, $P > 0.05$) (data not shown). Since P2Y receptors mobilize calcium from intracellular stores while P2X receptors use calcium from extracellular sources, the results with pre-exposure of cells to EGTA suggest that P2Y receptors were responding to UTP while ATP was having its effect mostly through P2X receptors.

Nucleotides enhance endocytosis and/or micropinocytosis

We next tested the effect of the nucleotides on the ability of the J774 cell line or primary peritoneal macrophages to internalize FITC-dextran. In J774 macrophages, incubation with 200 μM ATP, ADP and α,β-meATP induced an increase in dextran internalization (137 ± 13%, 117 ± 5%, 113 ± 4%, respectively), while BzATP had an inhibitory effect (58 ± 10%) at the concentration used (Fig. 2A). Neither UTP nor UDP had any effect on dextran internalization. Pre-incubation with the nucleotides had a larger effect on the uptake of dextran particles by peritoneal macrophages (Fig. 2B). The agonist α,β-meATP, at 10 μM, was the most powerful inducer of dextran uptake, increasing the uptake by 140 ± 38%, compared with untreated control cells (Fig. 2B). The same agonist had smaller effects at 1 or 100 μM, being 57 ± 20% and 50 ± 15%, respectively, while ATP (100 μM) and ADP (200 μM) increased dextran uptake

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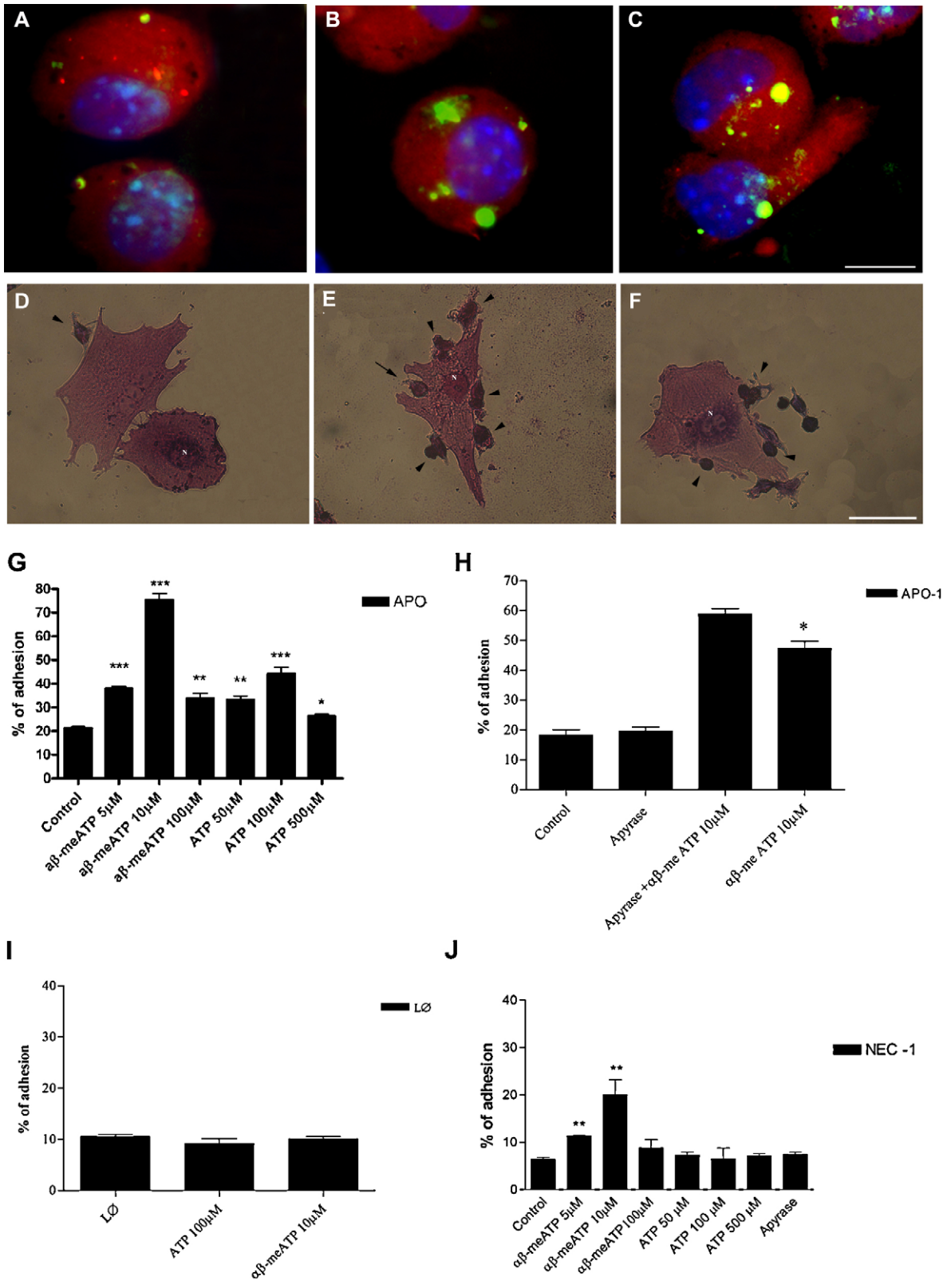

Fig. 5. α,β-meATP and ATP increase binding of apoptotic and necrotic but not viable cells to macrophages. (A–C) Fluorescence images (optical sections) of macrophages that had been exposed to apoptotic (APO-1) FITC-labelled cells were stained with Evans blue (red-cytoplasm) and DAPI (blue-nuclei) observed in the ApoTome microscope. (A) Control cells, (B) cells pre-treated with ATP, and (C) cells pre-treated with α,β-meATP. In panels (D–F), images were obtained by optical microscopy of apoptotic bodies (arrow heads) adhering to (D) untreated macrophages, or to macrophages that had been treated with (E) 100 μM α,β-meATP or (F) 100 μM ATP. Arrow heads correspond to apoptotic bodies, while the arrow refers to an apoptotic body at early stages of phagocytosis. Bar = 10 μm. Peritoneal macrophages were plated for 24 h; treated with α,β-meATP, ATP or control buffer; and then exposed to (G) apoptotic cells (APO-1), (I) viable lymphocytes (LØ), or (J) necrotic cells (NEC-1) for 3 h. In (H), macrophages were plated in the presence or absence of apyrase, treated with α,β-meATP, and then exposed to apoptotic cells (APO-1). Values represent the average and SEM of 3 separate experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

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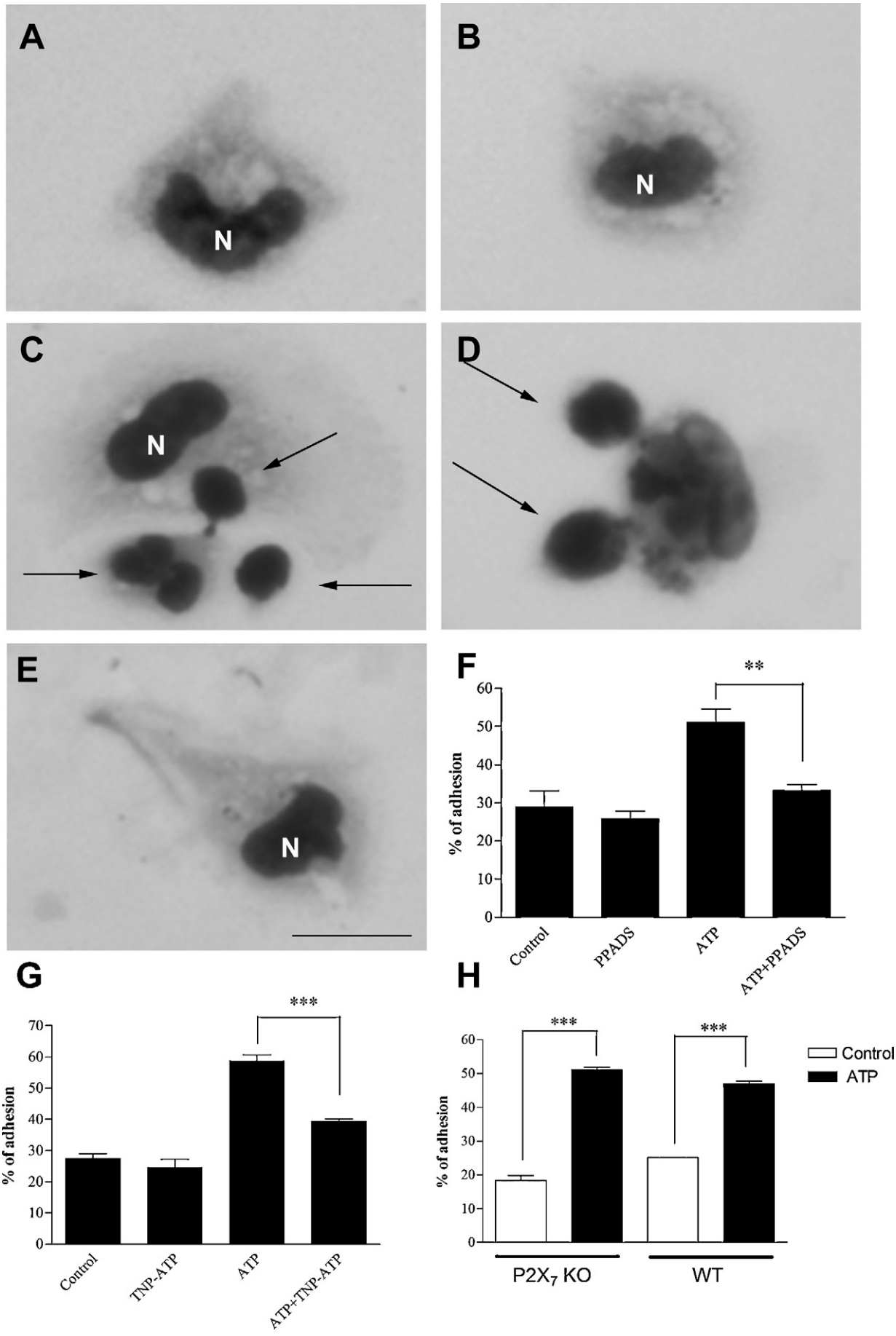

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by 71 ± 28% and 54 ± 17%, respectively, compared with control cells (Fig. 2B).

Nucleotides increase expression of CD11b, CD51 and CD61

We next determined whether the expression of macrophage surface receptors that are involved in the recognition and uptake of apoptotic cells might also be modulated by nucleotides. We therefore measured the expression of the integrins CD11b/Mac-1, α_v (CD51) and β_3 (CD61) by flow cytometry, as these are involved in either binding or internalization of apoptotic cells (Maderna and Godson 2003). In peritoneal macrophages, 10 μM α,β-meATP increased CD11b/Mac-1 expression by 26 ± 2%, and 100 μM ATP increased it by 22 ± 3% (Fig. 3E), as measured by flow cytometry (Fig. 3D). The treatment of cells with 100 μM ADP also increased CD11b expression, which was inhibited by pre-treatment of the cells with MRS2179 (data not shown). Immunofluorescence images also showed formation of membrane projections in peritoneal macrophages after treatment with 10 μM α,β-meATP (Fig. 3C), compared to untreated cells, which often are rounder (Fig. 3B). The changes in macrophage morphology after α,β-meATP treatment were confirmed by analysis of images obtained by fluorescence microscopy and actin staining (data not shown). Peritoneal macrophage expression of the integrins α_v (CD51) (Fig. 3F) and β_3 (CD61) (Fig. 3G) was also significantly enhanced by incubation with 10 μM α,β-meATP (54 ± 3% and 12 ± 2%) and 100 μM ATP (64 ± 3% and 26 ± 3%), but not 100 μM BzATP.

All of the P2 agonists, α,β-meATP, BzATP, ATP and ADP, at different concentrations, induced upregulation of CD11b in J774 macrophages (Fig. 4A–D). While α,β-meATP was the most effective nucleotide at lower concentrations (1 μM) (Fig. 4A), ATP and ADP were able to upregulate CD11b expression significantly at a concentration of 100 μM (Fig. 4C and D). At a higher concentration (500 μM), ATP had a negative effect, downregulating CD11b, probably by stimulating $P2X_7$-mediated cytotoxicity. BzATP was equally effective at 1, 10 and 100 μM (Fig. 4B). ADP similarly had a small, but significant, stimulatory effect at 100 μM (Fig. 4D).

Binding of apoptotic and necrotic cells to macrophages is increased by nucleotides

Since we observed enhanced expression of CD11b on macrophages and modulation of phagocytosis after nucleotide exposure, we next analyzed whether this may affect the ability of the macrophages to clear apoptotic cells (Fig. 5). Untreated macrophages can phagocytose some apoptotic bodies (Fig. 5A). However there was a marked increase in the phagocytosis of apoptotic cells and bodies after the macrophages were stimulated with 100 μM ATP (Fig. 5B) or 100 μM α,β-meATP (Fig. 5C). To quantify the ability of macrophages to clear apoptotic cells, we also evaluated the adhesion of macrophages to apoptotic cells. Untreated macrophages can bind some apoptotic cells (Fig. 5D). However, there was a marked increase in the adhesion of apoptotic cells to macrophages after the macrophages were stimulated with α,β-meATP (Fig. 5E) or ATP (Fig. 5F). The number of apoptotic cells (Apo-1) bound to macrophages (rosettes) was quantified by direct counting of optical micrographs, such as in Fig. 5G and H. Untreated macrophages displayed 21 ± 1% adhesion of apoptotic cells, but treatment with ATP at 50, 100 and 500 μM increased adhesion to 33 ± 2%, 44 ± 2%, and 26.4 ± 0.6%, respectively. The nucleotide α,β-meATP showed biphasic effects again at higher concentrations, inducing 38 ± 1% adhesion at 5 μM, 75 ± 3% at 10 μM, and 34 ± 2% at 100 μM (Fig. 5G). In some experiments, we collected and cultivated the macrophages without addition of apyrase in the medium, and observed a significant reduction in α,β-meATP-induced adhesion of apoptotic cells (Fig. 5H). We also used ADP as agonist followed by the adhesion assay but observed no changes in uptake of apoptotic cells in spite of the fact that ADP upregulated CD11b expression (data not shown). The nucleotides had no effect on adhesion of viable non-apoptotic cells, which was 10.5 ± 0.4% whether or not the macrophages were treated with nucleotides (Fig. 5I). In addition, the pre-treatment with CD11b neutralizing antibodies partially reversed ATP-induced adhesion of apoptotic cells (data not shown).

The nucleotides also affected adhesion of necrotic cells by macrophages, although not to such a high level as for apoptotic cells. Adhesion of necrotic cells (Nec-1) increased to 20 ± 3% after treatment of macrophages with 10 μM α,β-meATP, which was significantly higher than macrophages treated with control buffer or apyrase (Fig. 5J).

We tested the relevance of these results for clearance of apoptotic cells *in vivo* by injecting mice in peritoneum with ATP or control buffer for 30 min, followed by injection with Apo-1 cells. After 3 h, peritoneal cells were collected and adhesion of apoptotic cells was measured by microscopy as above. ATP treatment increased adhesion of Apo-1 cells by 25 ± 10%, compared to mice treated with control buffer (untreated mice) (Fig. 6).

Binding of apoptotic cells to macrophages by nucleotides is antagonized by P2X antagonists

We next evaluated which P2 receptors may be involved in the ATP-induced clearance of apoptotic cells *in vivo* by injecting mice in peritoneum with PPADS (non-selective P2X antagonist but not effective for $P2X_4$) and TNP-ATP (selective antagonist for $P2X_1$ and $P2X_3$) prior to ATP injection. We observed that both treatments completely antagonized the stimulatory effect of ATP (Fig. 6A–G). In addition, ATP was able to induce an increase in Apo-1 cell adhesion to peritoneal macrophages from both wildtype and $P2X_7$-deficient mice (Fig. 6H), indicating that several receptors are involved in the ATP-mediated effects.

Discussion

Based on the results with different P2 receptor agonists and antagonists used in this study, multiple P2 receptors on macrophages are most likely involved in the modulation of phagocytosis of apoptotic cells. At least two ATP-activated P2X receptors ($P2X_1$ and/or $P2X_3$) could account for the modulation of phagocytosis of apoptotic cells following treatment of macrophages with extracellular nucleotides. These two P2X receptors, $P2X_7$, and one ADP-activated receptor ($P2Y_1$) could contribute to the increase of phagocytosis of latex beads by macrophages. Recently, two papers have suggested that activation of $P2X_7$ receptors for 10–30 min can

Fig. 6. $P2X_1$ and $P2X_3$ receptor antagonists block the ATP-induced increase of adhesion of apoptotic bodies to macrophages. Swiss mice were injected in the peritoneum with control buffer (A), 500 μM PPADS (B, E and F) or 500 μM TNP-ATP (G) for 60 min followed by peritoneal injection of ATP (C–G) for an additional 30 min, and finally by injection of APO-1 cells. Images obtained by optical microscopy of apoptotic bodies (arrow) adhering to macrophages after ATP treatment (C and D). Wildtype or $P2X_7$-deficient mice were injected in the peritoneum with control buffer or ATP for 30 min followed by peritoneal injection of APO-1 cells (H). The adhesion index was determined by direct counting of peritoneal macrophages isolated from the mice. Three animals were used for each experimental condition. N = macrophage nucleus, arrows = apoptotic bodies, bar = 5 μm. Values represent the average and SEM of 3 separate experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$.

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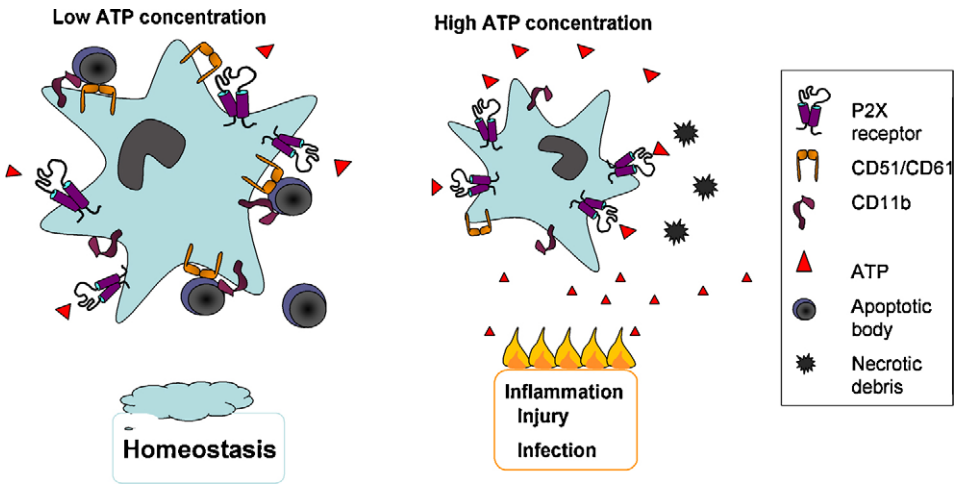

Fig. 7. Model of modulation of uptake of apoptotic cells by nucleotides. Cells in a homeostatic environment release low ATP concentrations, which stimulate macrophages to clear apoptotic bodies, due to P2X-mediated upregulation of the adhesion molecules CD11b and CD51/61. Conversely, necrotic cells release high concentrations of ATP, which do not induce clearance of the necrotic cells and instead stimulate inflammation.

downregulate phagocytosis of latex beads in microglia (Fang et al. 2009) and latex beads and bacteria in macrophages (Gu et al. 2009). Our data are partially in agreement with their results, since long exposure (30 min) of ATP or BzATP treatment reduced phagocytosis in macrophages (Fig. 1D). However short treatment with $P2X_7$ agonists (10 min) had the opposite effect, suggesting that response of $P2X_7$ receptors could depend of nucleotide concentration and time exposure.

The α,β-meATP agonist is specific for both $P2X_1$ and $P2X_3$ receptors, and we have previously reported the expression and function of $P2X_3$ receptors on macrophages (Coutinho-Silva et al. 2005). $P2X_1$ receptors are functional in macrophages only when the cells are isolated in the presence of the ATP-consuming enzyme, apyrase (Sim et al. 2007). Our data from Fig. 5H, showing that apyrase can modulate the α,β-meATP-induced increase of phagocytosis of apoptotic cells, favor the hypothesis that $P2X_1$ is the main P2X receptor involved. In addition, the partial dependence of extracellular calcium on ATP-induced modulation of phagocytosis suggested the participation of both P2X and P2Y receptors.

Recently, uridine nucleotides acting at $P2Y_6$ receptors were shown to mediate microglial phagocytosis (Koizumi et al. 2007). Here we extend these findings, demonstrating that UTP and UDP could also modulate phagocytosis in macrophages, most likely via $P2Y_6$ receptors. However, these findings will require further studies to firmly establish a role for uridine nucleotides in phagocytosis by macrophages.

As both apoptotic and necrotic cells likely co-exist in damaged or infected tissues, we propose that low concentrations of extracellular ATP could engage P2X (possibly $P2X_1$ or $P2X_3$) and/or $P2Y_1$ receptors, resulting in the increased expression of "eat-me" signals receptors on macrophages such as CD11b and $\alpha_v\beta3$ receptor and consequently, more efficient binding of apoptotic cells and bodies (Fig. 7). Consistent with this possibility, we recently observed that uptake of *Leishmania amazonensis* promastigotes (which are phosphatidylserine positive) by macrophages is higher when host macrophages are pre-treated with αβ-meATP. This suggests that upregulation of CD11b by αβ-meATP in host cells increases recognition of the parasites (our unpublished data). However, consistent with our results, the higher concentrations of ATP released in tissues undergoing high levels of necrosis should not stimulate upregulation of the CD11b and $\alpha_v\beta3$ receptors, nor clearance of necrotic cells, thus amplifying the inflammatory effects of debris released from necrotic cells.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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