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Adenosine A_3 receptors negatively regulate the engulfment-dependent apoptotic cell suppression of inflammation

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ABSTRACT

Timed initiation of apoptotic cell death followed by efficient removal mediated by professional macrophages is a key mechanism in maintaining tissue homeostasis. Besides phagocytosis, clearance of apoptotic cells also involves suppression of inflammatory responses by apoptotic cells mediated by both direct inhibition of pro-inflammatory cytokine production and release of soluble anti-inflammatory factors, which act in a paracrine or autocrine fashion to amplify or sustain the anti-inflammatory response. Previous work has demonstrated that during engulfment of apoptotic cells adenosine is produced in sufficient amounts to trigger both adenosine A_{2A} receptors (A2ARs) and A_3 receptors (A3Rs). Adenosine bound to A2ARs of macrophages activated the adenylate cyclase pathway to suppress the apoptotic-cell induced, NO-dependent formation of neutrophil migration factors. Here we show by using A3R null engulfing macrophages that the adenosine produced triggers the A3Rs as well, which attenuate the A2AR signaling by inhibiting adenylate cyclase. As a result, the balance in the activation of A2ARs and A3Rs determines the amounts of NO and consequently the levels of neutrophil chemoattractants formed. Since during phagocytosis of apoptotic cells the expression of A2ARs increases, while that of A3Rs decreases, on long term adenosine suppresses the proinflammatory responses in engulfing macrophages.

1. Introduction

Most cell types have a limited life span, which ends physiologically through the process of apoptosis, or programmed cell death. *In vivo*, apoptotic cells are usually engulfed by neighboring cells or professional phagocytes, such as macrophages to reutilize their materials and to prevent the release of their intracellular cell content which could harm the surrounding tissues [1]. While the phagocytosis of a variety of pathogenic targets, especially bacteria and virally-infected cells, normally triggers a pro-inflammatory response in macrophages (including the generation of reactive oxygen-derived intermediates, the release of proteolytic enzymes, and the production of numerous inflammatory cytokines), ingestion of apoptotic cells by macrophages usually induces an anti-inflammatory phenotype. Apoptotic cells do not necessarily fail to provide pro-inflammatory signals; rather, they can initiate signaling pathways in macrophages, which can actively interfere with the inflammatory program. For example, preincubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced *via* Toll-like receptor 4 by lipopolysaccharide, a component of the cell wall of Gram-negative bacteria [2–4]. This inhibitory property appears to be a common attribute acquired post-translationally by all cells undergoing apoptotic cell death, regardless of the cell type or the particular death stimulus [4–6].

The mechanism(s) by which apoptotic cells exert their inhibition on phagocytes may vary over time. The earliest anti-inflammatory activity of the apoptotic cell is manifest as an immediate-early inhibition of macrophage pro-inflammatory cytokine gene transcription and is exerted directly upon binding to the macrophage, independent of subsequent engulfment and soluble factor involvement [4]. Subsequently, soluble mediators are released from macrophages, which act in a paracrine or autocrine fashion to amplify and sustain the anti-inflammatory response [2,3,7].

Adenosine is a purine nucleoside that, following its release from cells or after being formed extracellularly, diffuses to the cell membrane of surrounding cells, where it binds to its receptors [8,9]. There are four adenosine receptors, all of which are G protein-coupled receptors and are abundantly expressed by macrophages

Abbreviations: A2AR, adenosine A_{2A} receptor; A3R, adenosine A_3 receptor; KC, cytokine-induced neutrophil-attracting chemokine; L-NAME, L-(G)-nitro-L-arginine methyl ester; MIP, macrophage inflammatory protein.

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[10]. The genes for these receptors have been analyzed in detail and are designated A_1, A_{2A}, A_{2B} and A_3. Adenosine A_1 receptors are stimulated by 10^{-10}–10^{-8} M concentrations of adenosine and mediate decreases in intracellular cyclic AMP (cAMP) levels, adenosine A_{2A} (A2AR) and A_{2B} receptors are stimulated by higher (5×10^{-7} M and 1×10^{-5} M, respectively) concentrations of adenosine and mediate increases in cAMP levels, while adenosine A_3 receptors (A3Rs) are stimulated by 10^{-6} M concentrations of adenosine and mediate adenylate cyclase inhibition [9].

Recent work in our laboratory has demonstrated that adenosine is one of the soluble factors produced by macrophages engulfing apoptotic cells, which is involved in the down-regulation of pro-inflammatory responses [11]. Adenosine bound to the A2ARs prevents the apoptotic cell-induced NO formation and the consequent NO-dependent neutrophil chemoattractant production in engulfing macrophages by activating the adenylate cyclase/protein kinase A pathway. Since adenosine was found to be produced in a sufficient amount to activate simultaneously both A2ARs and A3Rs [11], in the present work the effect of A3Rs was studied on the pro-inflammatory cytokine formation of macrophages engulfing apoptotic cells by studying the apoptotic cell-induced suppression of proinflammatory cytokine formation in macrophages isolated from A3R null mice [12].

2. Materials and methods

2.1. Reagents

All reagents were obtained from (Sigma–Aldrich, Budapest) except indicated otherwise.

2.2. Animals

The experiments were done using 3 months old wild type, adenosine A_3 receptor deficient mice developed and provided to us by the Merck Co., NJ, USA [12] generated on an a C57Bl/6 background. Some of the studies were carried out on adenosine A_{2A} receptor deficient mice [13] generated on an FVB background. The studies presented have been reviewed and approved by the review committee of the University of Debrecen [DEMÁB].

2.3. Macrophage isolation and culturing

Macrophages were obtained by peritoneal lavage with sterile physiological saline. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 for 2 days before use. After 3–4 h incubation, the non-adherent cells were washed away. Before the experiments the cells were cultured for 2 days replacing media daily.

2.4. Thymocyte apoptosis induction in vitro

Apoptotic cells were prepared from 4 weeks old wild-type or pannexin null mice [24]. The isolated thymocytes isolated were cultured for 24 h (10^7 cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. This method typically resulted in >80% apoptotic cells (as assessed by propidium iodide/AnnexinV-FITC staining) [11]. Apoptotic cells were used at a 10:1 (apoptotic cell:macrophage) ratio.

2.5. Determination of adenosine A_3 receptor expression on the cell surface

Wild type and A3R null peritoneal macrophages were coincubated with apoptotic thymocytes for 1 h in 1:10 ratio. After replacing media and washing away the apoptotic cells, macrophages were incubated for additional 1, 3 or 5 h. After the treatments macrophages were washed (1× PBS), collected, blocked with 50% FBS for 30 min and labeled with anti-mouse A3R antibody (Santa Cruz Biotechnology) or goat IgG isotype control (R&D Systems). For the detection cells were stained with FITC-conjugated anti-goat IgG. Stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

2.6. Determination of adenosine A_3 receptor mRNA expression

Wild-type peritoneal macrophages were coincubated with various target cell types: apoptotic, living, heat killed (45 min, 55 °C) or anti-CD3-pretreated (10 μg/ml, 20 min; R&D Systems) adenosine A3 null thymocytes for 1 h in 1:10 ratio. After washing away the apoptotic cells and replacing media mRNA was collected 2 h later.

2.7. Determination of adenosine A_3 receptor protein levels

Peritoneal macrophages were homogenized in ice-cold lysis buffer containing 0.5% Triton X-100. Protein concentration of samples was diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. Electrophoresis was performed in 10% SDS-polyacrylamide gel. Separated proteins were transferred to an Immobilion-P transfer membrane (Millipore, Budapest, Hungary) and were probed with anti-mouse A3R antibody (Santa Cruz Biotechnology). To detect A3R signals HRP-labeled anti-goat antibodies were used. Protein bands were visualized by Immobilon Western Chemiluminescent HRP substrate (Millipore, Budapest, Hungary).

2.8. Determination of cytokine production

Wild-type and A3R null peritoneal macrophages were plated onto 24-well plates at a density of 1×10^6 cells/well. To determine cytokine production by macrophages exposed to apoptotic cells, macrophages ($A3R^{+/+}$ or $A3R^{-/-}$) were exposed to apoptotic cells for 1 h in the presence or absence of the A3R-selective agonist IB-MECA (10 μM) or the A3R-selective inhibitor MRS1523 (10 μM). Apoptotic cells than were washed away, the compounds were readded and the macrophages were cultured for an additional 5 h. At the end of culture cell culture media were analyzed by Mouse Cytokine Array (Proteome Profile Array from R&D Systems). The pixel density in each spot of the array was determined by Image J software. Alternatively, cytokine-induced neutrophil-attracting chemokine (KC), and macrophage inflammatory protein-2 (MIP-2) cytokine levels were measured with R&D Systems ELISA kits.

2.9. Phagocytosis assay

For visualizing apoptotic cells in macrophages, macrophages were plated in 2-well chamber slides in a concentration of 5×10^5/well and cultured for 48 h before staining overnight with10 μM 5-(and 6-)-(((4-chloromethyl) benzoyl)amino)tetramethylrhodamine (CMTMR) (Invitrogen), while thymocytes were labeled overnight with 6 μM 6-carboxy-3′,6′-diacetylfluorescein (CFDA) (Invitrogen). After coculturing macrophages with CFDA labeled apoptotic cells for 30 min, cells were washed and fixed in ethanol/acetone (1:1) for 10 min at −20 °C. Images were taken with an Olympus FV1000 confocal laser scanning microscope. Five hundred cells were counted for apoptotic cell uptake in each individual experiment.

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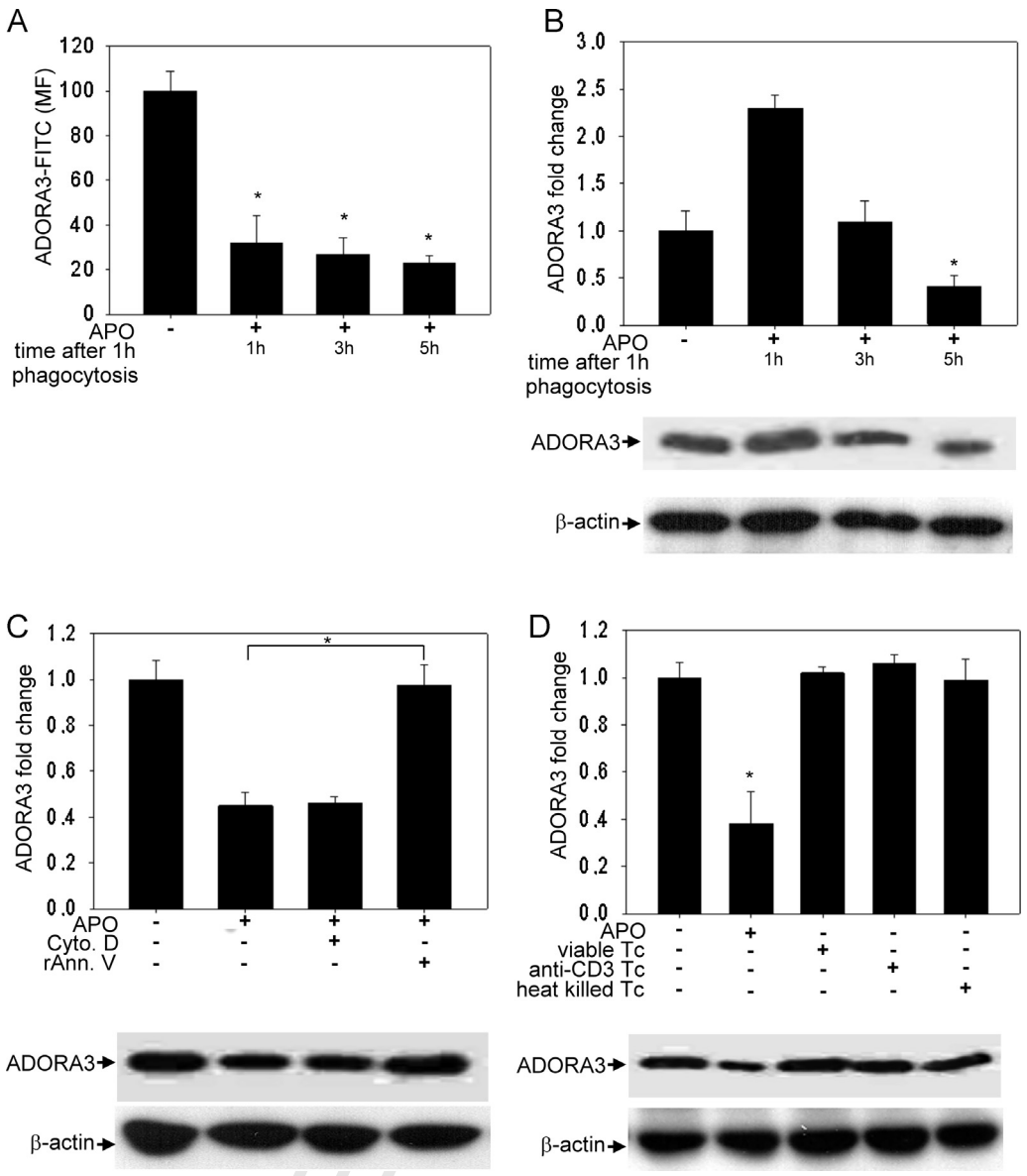

Fig. 1. Apoptotic cell recognition inhibits the cell surface expression and the *de novo* adenosine A_3 receptor synthesis in peritoneal macrophages. Wild-type peritoneal macrophages were exposed for 1 h to A3R null apoptotic thymocytes and cell surface A3R (A) and A3R mRNA and protein levels (B) were determined by flow cytometry, quantitative PCR and Western Blot, respectively, at the indicated time periods following washing away of apoptotic cells. (C) Blocking of apoptotic cell recognition abolishes the decrease of A_3 receptor level. Phagocytosis was inhibited by pretreating macrophages with 50 mM cytochalasin D or by masking the phosphatidylserine on the apoptotic cell surface with recombinant annexin V (10 μg/10^5 apoptotic cell). After 1 h co-incubation apoptotic cells were washed away and macrophages were further cultured for 3 h. Adenosine A_3 receptor mRNA and protein levels were determined by quantitative PCR and Western Blot, respectively. (D) Only apoptotic cell recognition decreases the *de novo* adenosine A_3 receptor synthesis in peritoneal macrophages. Macrophages were coincubated with apoptotic, living, heat killed (45 min, 55 °C) or anti-CD3-pretreated (10 μg/ml, 20 min) A3R null thymocytes for 1 h. Thymocytes were then washed away and macrophages were further incubated for 3 h. The gene expression and protein levels were measured by quantitative PCR and Western Blot, respectively. MFI: mean fluorescence intensity. Results are expressed as mean ± SD of three or four independent experiments (*$P < 0.05$).

2.10. Determination of NO production of macrophages engulfing apoptotic cells

Wild-type or A3R null macrophages were exposed to apoptotic cells for 1 h. Media were replaced and macrophages were incubated for an additional 1 h. Cell culture supernatants were analyzed for NO production by measuring nitrite, a stable oxidation product of NO, using the Griess–Ilosvay method [14].

2.11. Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± SD. *P* values were calculated by using two-tailed Student's *t*-test for two samples of unequal variance. The analysis of cytokine array experiments was carried out by ANOVA test. Statistical significance is indicated by a single asterisk ($P < 0.05$).

3. Results

3.1. The expression of adenosine A_3 receptors is decreased on the cell surface of macrophages during engulfment of apoptotic cells

Previous studies have shown that adenosine A3Rs are expressed by macrophages and contribute to the directed migration of these cells [15]. Since our studies have shown that macrophages

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A

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	+ control											+ control
B	BLC	C5a	G-CSF	GM-CSF	I-309	Eotaxin	sICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12 p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1α	MIP-1β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF-α	TREM-1								
F	+ control											- control

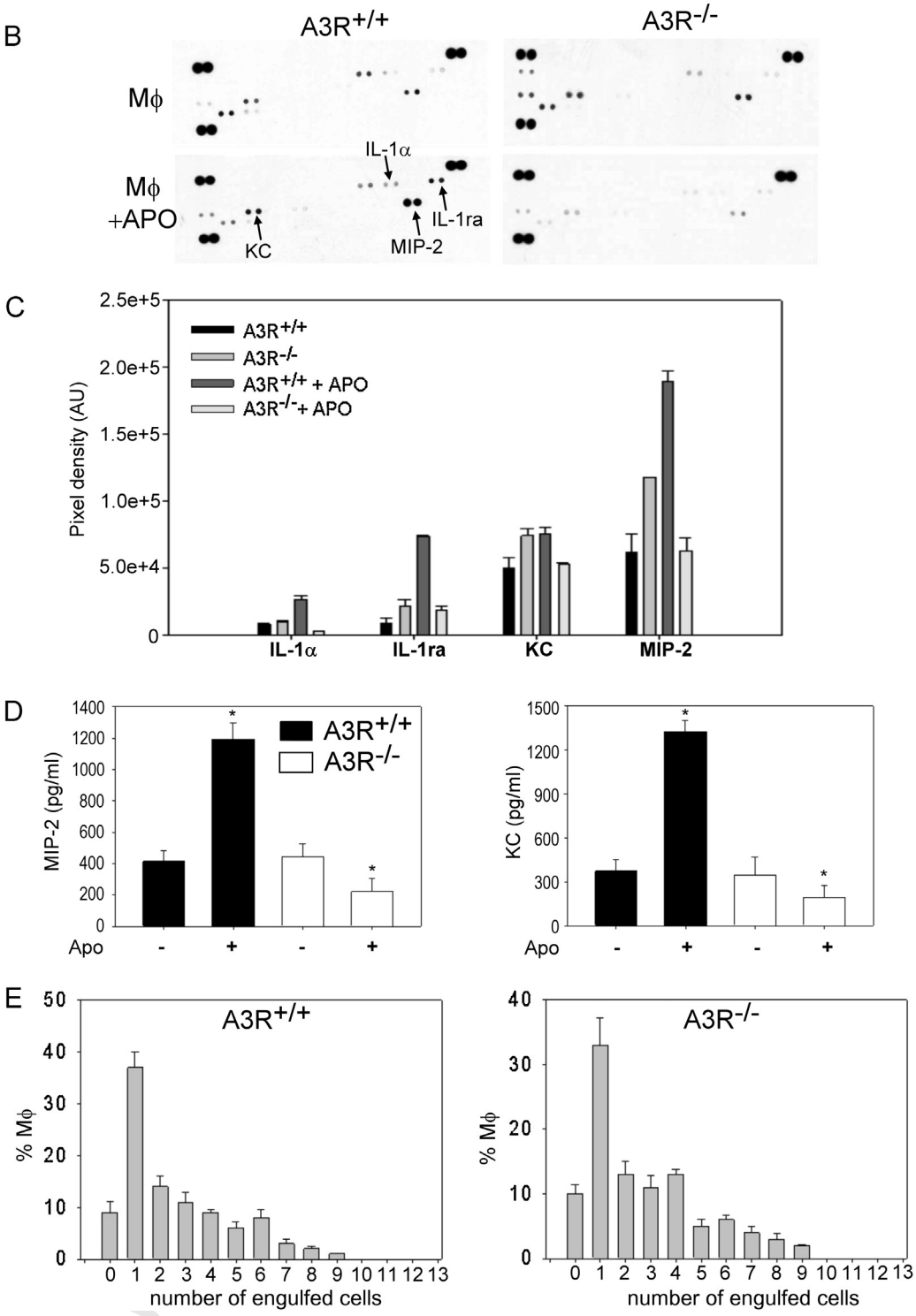

Fig. 2. Adenosine A_3 receptor-deficient macrophages respond to apoptotic cells with decreased MIP-2 and KC production. (A) The map of the 40 cytokines detected on the membranes. (B) Cytokine panel of control and apoptotic cell treated wild-type and adenosine A_3 receptor null peritoneal macrophages. Peritoneal macrophages were co-incubated with apoptotic thymocytes for 1 h (MPh:APO = 1:10) followed by removal of apoptotic cells and addition of fresh medium. Supernatants were collected 5 h later and cytokine levels were determined by cytokine array. Arrows highlight KC, MIP-2, IP-10, IL-1 and IL-1ra neutrophil chemoattractants, the production of which is significantly downregulated by A3R null macrophages. (C) Cytokines, which levels were significantly different ($P < 0.05$) in the supernatants analyzed by cytokine array. (D) Wild-type and A3R null macrophages were exposed to apoptotic thymocytes for 1 h (MPh:APO = 1:10). 5 h after the removal of apoptotic cells supernatants were collected. The levels

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engulfing apoptotic cell produce adenosine and express increasing levels of A2ARs [11], we decided to test whether the expression levels of the A3R are altered during phagocytosis of apoptotic cells. As shown in Fig. 1A, we could confirm the expression of the cell surface adenosine A_3 receptors in mouse peritoneal macrophages, but this expression is significantly decreased following incubation with apoptotic cells. The inhibition partially involved transcriptional regulation, since not only the cell surface expression of A3R, but its mRNA and total protein levels also decreased during long term phagocytosis (Fig. 1B). However, the decrease in the cell surface expression could be detected much earlier than that of the mRNA and the protein, indicating that fast posttranslational mechanisms might also be involved in the downregulation of A3R.

Cytochalasin D does inhibit the engulfment process, but it does not influence the recognition of apoptotic cells [4]. Binding of phosphatidylserine on the surface of apoptotic cells plays a key role in their recognition and subsequent uptake by macrophages, and this recognition can be inhibited by preincubation of apoptotic cells with recombinant annexin V (which binds to phosphatidylserine; ref. 16). While recombinant annexin V prevented the downregulation of A3R mRNA and protein expression by apoptotic cells (Fig. 1C), cytochalasin D had no effect on it, suggesting that not the engulfment, but the recognition *per se* triggers the decrease in the A3R mRNA and protein expression. Inhibition of A3R expression during phagocytosis was specific for the engulfment of apoptotic cells, as uptake of neither necrotic nor antibody-coated cells affected it (Fig. 1D).

3.2. Loss of adenosine A_3 receptor influences the pro-inflammatory cytokine production by macrophages engulfing apoptotic cells

Evaluation of the cytokine secretion profile of unstimulated macrophages was performed using a highly sensitive cytokine antibody array method, enabling the simultaneous detection of low concentrations of multiple cytokines in one assay (picogram per milliliter range). The map of the 40 cytokines detected on the membranes is diagrammed in Fig. 2A. The cytokines in our experimental systems were first evaluated by experiments using untreated wild-type and A3R$^{-/-}$ macrophages *in vitro*. The results reported in Fig. 2B show that the loss of the A3R did not affect significantly the composition of the cytokines released. In the levels of the cytokines we found individual animal variations, but when the MIP and KC levels were determined in many samples by ELISA, no significant difference could be detected in their basal levels when we compared the wild type and the A3R$^{-/-}$ (Fig. 2D). However, as shown in Fig. 2C, when macrophages were exposed to apoptotic cells, we found four cytokines whose production was increased by wild-type macrophages engulfing apoptotic cells compared to their non-engulfing counterparts, but decreased in A3R$^{-/-}$ macrophages. These cytokines include the cytokine-induced neutrophil-attracting chemokine (KC), and the macrophage inflammatory protein-2 (MIP-2), which act as chemoattractants for neutrophils and/or other cell types [17–19], the pro-inflammatory cytokine IL-1α [20] and the antagonist of the IL-1 receptor (IL-1ra). The modification in the levels of these cytokines was not due to a difference in the extent of phagocytosis, as a similar rate of engulfment by wild-type and A3R$^{-/-}$ macrophages was observed by counting the number of apoptotic cells taken up by 500 individual macrophages (Fig. 2E). Among the cytokines released in altered amounts in the supernatant by cultured A3R$^{-/-}$ macrophages, MIP-2 showed the most dynamic change in response to apoptotic cell exposure, and MIP-2 and KC levels were detected in the highest amounts (Fig. 2B and C). These observations correlate with those found in the A2AR null macrophages, in which the loss of the A2AR affected the production of the same cytokines in the presence of apoptotic cells [11], but the direction of the alteration was the opposite: the loss of A2ARs lead to an increase in the production of these cytokines. To confirm further the effect of A3Rs on the expression of the above cytokines during apoptotic cell engulfment, we decided to test the effect of A3R on the pro-inflammatory cytokine production by exposing wild-type macrophages either to the highly specific A3R agonist IB-MECA or to the A3R antagonist MRS1523. As shown in Fig. 3, using wild-type macrophages we could confirm the decreased expression of the proinflammatory cytokines in the presence of the A3R antagonist, indicating that altered cytokine response of engulfing A3R null macrophages is not due to an altered differentiation of the cells. In addition, the presence of the A3R agonist enhanced the expression of these cytokines indicating that A3R signaling indeed negatively regulates the engulfment-dependent apoptotic cell suppression of inflammation, at least if these cytokines are concerned. The same compounds had no effect on the apoptotic cell-induced proinflammatory cytokine production, when they were administered to A3R$^{-/-}$ macrophages (data not shown) indicating that they indeed act *via* the A3Rs. To confirm the results, MIP-2 and KC protein levels also were assessed by ELISA. In harmony with the cytokine array results, while the production of MIP-2 and KC was enhanced by wild-type macrophages exposed to apoptotic cells as compared to non-engulfing macrophages, the release of both MIP-2 and KC by A3R$^{-/-}$ macrophages was decreased under the same conditions (Fig. 2D).

3.3. The apoptotic cell-mediated decrease in MIP-2 and KC production by A3R$^{-/-}$ macrophages is related to an enhanced protein kinase A signaling by A2ARs

Since among the neutrophil chemoattractants, the release of which was simultaneously altered by the loss of both the A2AR and the A3R, the production of MIP-2 and KC was studied in our previous experiments in details, we decided to investigate further the regulation of the expression of these two cytokines. Previously we have shown [11] that the production of these two cytokines would be triggered by the uptake of apoptotic cells, but simultaneous activation of the adenylate cyclase pathway triggered by the A2ARs attenuated the response. Since A3Rs are known to inhibit adenylate cyclase activity *via* stimulating Gi [9], we speculated that if adenosine is produced by engulfing macrophages in an amount that can trigger A3Rs, loss of A3R signaling would lead to enhanced adenylate cyclase signaling by A2ARs in A3R null macrophages. If it is so, enhanced adenylate cyclase signaling could explain the more effective inhibition of KC and MIP-2 production by engulfing A3R null macrophages. Indeed, addition of SCH442416, a selective A2AR antagonist, H89, a protein kinase A inhibitor, or Rp-cAMPS triethylamine, a specific membrane-permeable inhibitor of activation by cAMP of cAMP dependent protein kinase I and II [21], all significantly enhanced the production of MIP-2 and KC in apoptotic cell-triggered A3R null macrophages (Fig. 4A). In contrast, addition of forskolin, an adenylate cyclase activator [22] decreased the MIP-2 and KC production in wild-type macrophages. The same concentration of forskolin, however, was ineffective in

of MIP-2 and KC were determined by ELISA. Results are expressed as mean ± SD of 10 independent experiments (*$P < 0.05$). (E) The number of engulfed fluorescently labeled apoptotic cells within WT or A3R-null macrophages counted by confocal microscopy following 1 h of phagocytosis. Results are expressed as mean ± SD of three independent experiments.

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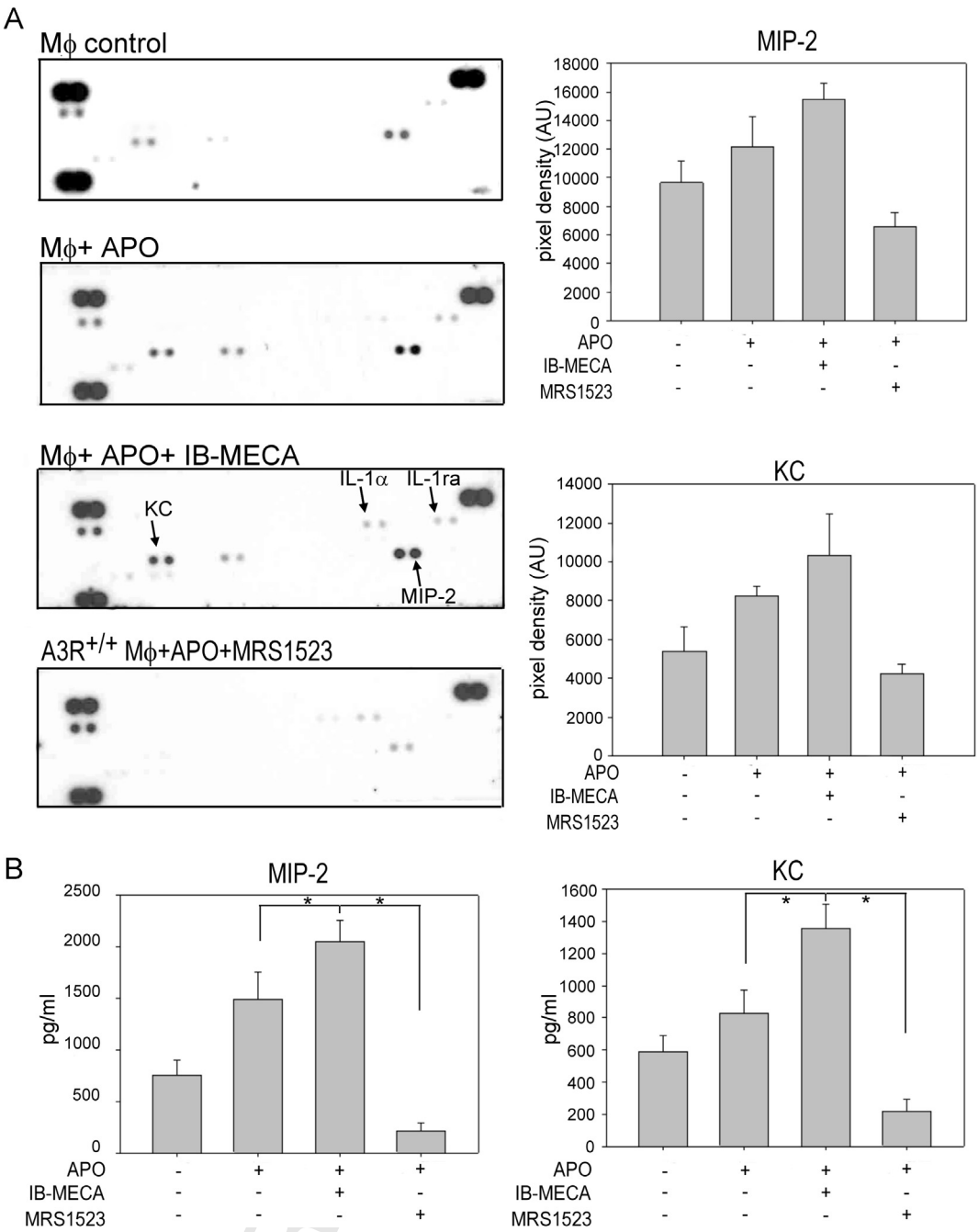

Fig. 3. As compared to controls, MIP-2 and KC production is enhanced by A3R agonist-treated, while decreased by A3R antagonist-treated wild-type macrophages engulfing apoptotic cells. (A) Cytokine panel of control, apoptotic cell exposed non-treated, IB-MECA (A3R-specific agonist)-treated or MRS1523 (A3R-specific antagonist)-treated wild-type macrophages. (B) MIP-2 and KC production of control, apoptotic cell exposed non-treated, IB-MECA (A3R-specific agonist)-treated or MRS1523 (A3R-specific antagonist)-treated wild-type macrophages determined by ELISA. Peritoneal macrophages were pre-incubated with 10 μM IB-MECA or with 10 μM MRS1523 for 30 min, then they were exposed to apoptotic thymocytes for 1 h (MPh:APO = 1:10). 5 h after the removal of apoptotic cells supernatants were collected and cytokine levels were determined by cytokine array. Arrows highlight KC, MIP-2 and IL-1β overproduced by IB-MECA-treated macrophages. Results are expressed as mean ± SD of three independent experiments (*$P < 0.05$).

A3R null cells indicating that adenylate cyclase is fully activated in these cells. When the adenylate cyclase pathway inhibitor Rp-cAMPS triethylamine was applied to A2AR or A3R null macrophages and their wild-type counterparts, a similar MIP-2 and KC production was found in the paired macrophages indicating that in the absence of adenylate cyclase signaling, the difference in apoptotic cell triggered-MIP-2 and KC production disappears (Fig. 4C). At the same time a significant strain difference was detected in the apoptotic cell-induced pro-inflammatory cell response. However, because at the moment we do not know how apoptotic cells induce the adenosine-controlled pro-inflammatory response, we cannot identify the molecular determinants for the observed difference between the mouse strains. All together these data indicate that in wild-type macrophages the adenylate cyclase pathway is not fully activated, and a balance in the A2AR and A3R signaling, which regulates the activity of the adenylate cyclase pathway in an opposite way, will decide the outcome of MIP-2 and KC production in the presence of apoptotic cells.

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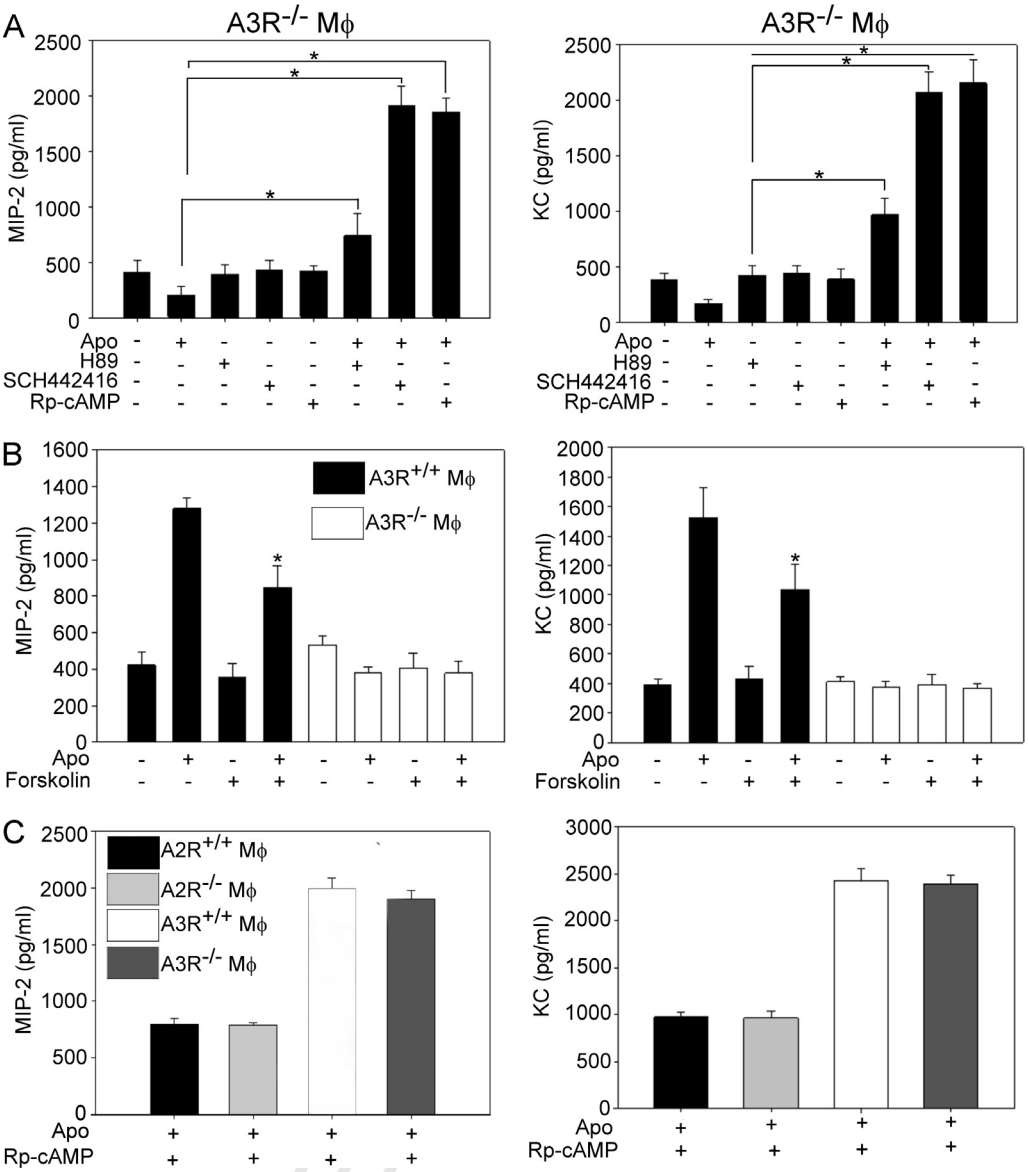

Fig. 4. Altered cytokine production of A3R null engulfing macrophages is related to an enhanced adenylate cyclase signaling induced by A2ARs. (A) A3R null macrophages were preincubated in the presence of 10 nM SCH442416, an A2A receptor antagonist, 100 μM Rp-cAMP triethylamine or 10 μM H89, inhibitors of protein kinase A for 30 min then exposed to apoptotic thymocytes for 1 h (MPh:APO = 1:10). (B) Wild type and A3R null macrophages were preincubated in the presence of 10 μM forskolin, an adenylate cyclase activator for 30 min, than exposed to apoptotic thymocytes for 1 h (MPh:APO = 1:10). (C) A3R and A2AR null macrophages and their wild-type counterparts were preincubated in the presence of 100 μM Rp-cAMP triethylamine for 30 min, than exposed to apoptotic thymocytes for 1 h (MPh:APO = 1:10). 5 h after the removal of apoptotic cells supernatants were collected in all the above experiments and the levels of MIP-2 and KC were determined by ELISA. The different basal levels of cytokines might be related to the fact that the mice were on different genetic background. Results are expressed as mean ± SD of three independent experiments (*$P < 0.05$).

3.4. Altered MIP-2 and KC production by A3R null macrophages is a consequence of altered NO production

Our previous studies have shown that upregulation of NO production in macrophages exposed to apoptotic cells contributes to MIP-2 release [11]. To confirm that regulation of NO production is involved in the altered MIP-2 and KC response of A3R null macrophages as well, macrophages were exposed to the nitric oxide synthase (NOS) inhibitor L-(G)-nitro-L-arginine methyl ester (L-NAME) before the addition of apoptotic cells. While, as we previously reported [11], addition of L-NAME attenuated apoptotic cell-induced MIP-2 protein expression by wild type macrophages (Fig. 5A), L-NAME had no effect on the MIP-2 production produced by A3R null macrophages. In accordance with these results, wild-type macrophages produced detectable NO levels upon apoptotic cell exposure, while A3R null macrophages did not (Fig. 5B). In the presence of RpcAMP, however, apoptotic cell exposed A3R$^{-/-}$ macrophages were capable of producing similar amounts of NO as wild-type macrophages indicating that their NO production is suppressed by the adenylate cyclase pathway. Previous studies have shown that addition of sodium nitropusside, a potent NO donor, alone did not induce MIP-2 production, but significantly enhanced apoptotic cell-induced MIP-2 production in wild-type macrophages [11]. Interestingly, sodium nitropusside, had no effect on MIP-2 production in A3R null engulfing macrophages (Fig. 5C). When, however, sodium nitropusside was applied to them together with RpcAMP, an enhanced MIP-2 production could be detected by A3R$^{-/-}$ engulfing macrophages as well. These data indicate that enhanced adenylate cyclase signaling in A3R null macrophages must inhibit simultaneously not only NO production, but also other signaling element(s) which contribute to the apoptotic cell-induced MIP-2 production.

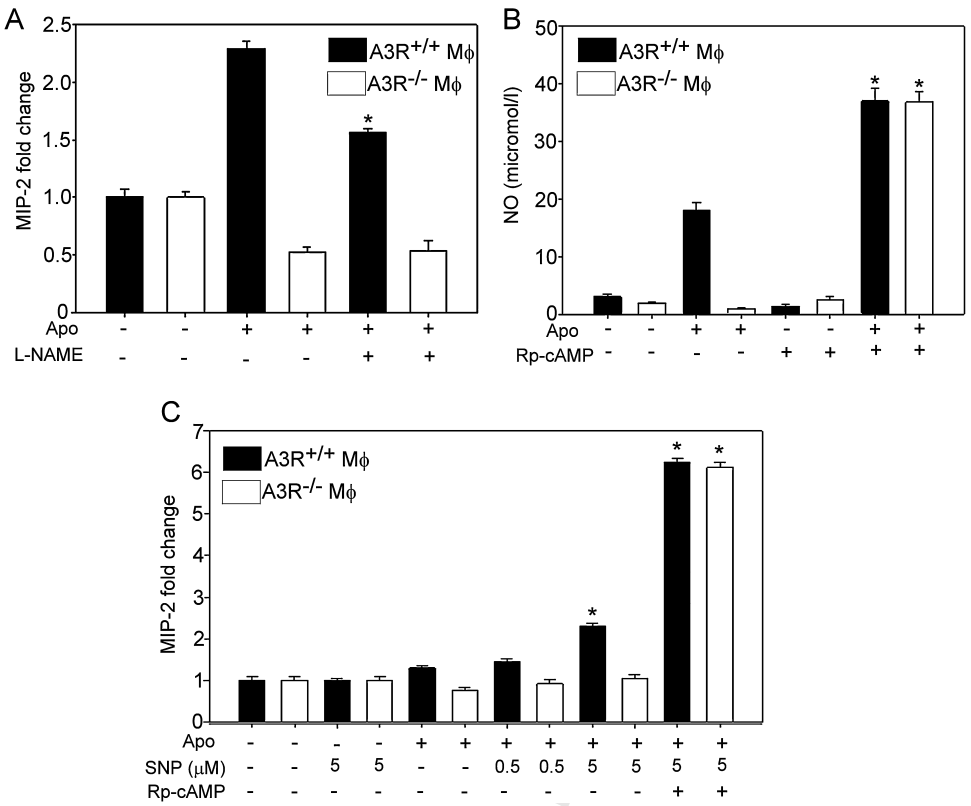

Fig. 5. Altered MIP-2 production by A3R null macrophages engulfing apoptotic cells is related to an altered NO production. (A) L-NAME, an inhibitor of nitric oxide synthetase, prevents apoptotic cell-induced MIP-2 production in wild-type engulfing macrophages, but has no effect on it in A3R null engulfing macrophages. Macrophages were preincubated in the presence of 5 μM L-NAME for 30 min prior to exposure to apoptotic cells for 1 h. MIP-2 mRNA levels were determined 2 h after the removal of apoptotic cells. (B) Engulfing A3 null macrophages do not produce NO, unless the adenylate cyclase pathway is inhibited. Macrophages were preincubated with 100 μM Rp-cAMP triethylamine for 30 min, than exposed to apoptotic thymocytes for 1 h (MPh:APO = 1:10). NO levels were determined 1 h later. (C) Exogenously produced NO stimulates apoptotic cell-triggered MIP-2 production in A3R null macrophages only if the adenylate cyclase pathway is simultaneously inhibited. Wild type and A3R null peritoneal macrophages were exposed to the indicated concentrations of the NO donor sodium nitropusside in the presence or absence of apoptotic cells. In some cases macrophages were pretreated with 100 μM Rp-cAMP triethylamine for 30 min prior to exposure to apoptotic cells. Apoptotic cells were washed away after 1 h. MIP-2 mRNA levels were determined 2 h later. Results are expressed as mean ± SD of five independent experiments (*$P < 0.05$).

4. Discussion

Acute inflammation normally resolves by preprogrammed mechanisms which are initiated in the first few hours after an inflammatory response begins. Among many others, these mechanisms involve phagocytosis of apoptotic neutrophils by macrophages leading to clearance of the dead neutrophils and release of anti-inflammatory and reparative cytokines [23]. We have shown recently that adenosine is one of the soluble mediators released upon engulfment, which contributes to the engulfment-dependent apoptotic cell suppression of inflammation [11]. Engulfing macrophages enhance their expression of A2ARs. Adenosine bound to A2ARs attenuates the apoptotic cell-induced NO formation and the consequent neutrophil chemoattractant induction, such as MIP-2 and KC, by activating the adenylate cyclase pathway. As a result, in an *in vivo* peritonitis model in the absence of A2AR signaling, $A2AR^{-/-}$ macrophages engulfing apoptotic cells produced enhanced amounts of MIP-2 and KC, and this was accompanied by a MIP-2- and KC-dependent neutrophil migration which was not seen in wild-type mice. Since adenosine was produced by engulfing macrophages in sufficient amount to trigger A3Rs as well [11], in the present study we investigated whether the A3R could be involved in the regulation of the anti-inflammatory response induced in macrophages by apoptotic cells. Here we report that in contrast to A2ARs engulfing macrophages downregulate A3Rs during phagocytosis. While upregulation of A2ARs involves activation of the nuclear lipid sensing receptors following phagocytosis [11], expression of A3Rs seems to be downregulated *via* cell surface macrophage receptors recognizing phosphatidylserine on the surface of apoptotic cells. Interestingly, while the cell surface expression of A3Rs decreased within 1 h, both its mRNA and protein levels increased first and then they decreased. The mechanism of this transient upregulation was not investigated in our study, but A3Rs were shown to be required for proper macrophage chemotaxis [24]. It is interesting to speculate that one of the apoptotic cell released 'find me' signals promote the expression of A3Rs during migration, while interaction with the apoptotic cells results in a fast disappearance of the receptor from the cell surface. During the past decade a number of macrophage phagocyte receptors were identified, which recognize phosphatidylserine either directly like BAI, TIM-4 and stabilin-2, or indirectly by using bridging molecules such as integrin-β3, which utilizes MFG-8, or Mer tyrosine kinase, which utilizes Gas6 for phosphatidylserine binding [25]. Thus triggering any of these receptors alone or in synchrony might initiate the downregulation of A3Rs during phagocytosis of apoptotic cells. Similar to A2ARs, loss of adenosine A3Rs did not affect the rate of phagocytosis. However, when exposed to apoptotic cells, $A3R^{-/-}$ macrophages notably produced decreased amounts of MIP-2 and KC acting as chemoattractants for various cell types, especially for neutrophils. We could confirm these data by using a specific A3R antagonist indicating that lack of the actual A3R signaling rather than altered macrophage differentiation in the absence of A3R explains the phenomenon. Interestingly, the production of a similar set of cytokines, namely the neutrophil

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chemoattractants, was affected by the loss of A3Rs as that of the loss of A2ARs, but in an opposite direction indicating that A3R receptor signaling in wild-type macrophages might inhibit the A2AR signaling. Indeed, the data presented indicate that in A3R null macrophages the loss of A3Rs, which would normally negatively regulate the adenylate cyclase activity during engulfment [9], results in an enhanced adenylate cyclase signaling by A2ARs leading to full suppression of the apoptotic cell-induced NO production and the consequent neutrophil chemoattractant formation. As a result, neither addition of the forskolin, which could further activate adenylate cyclase, nor inhibition of NO synthesis, since no NO production was detected, had an effect on the amount of MIP-2 produced by A3R null macrophages engulfing apoptotic cells. Previous studies have shown that ingestion of apoptotic cells is accompanied also by a release of PGE_2 [3] which acting *via* EP_{2-4} receptors [26] could also activate adenylate cyclase. However, when we inhibited adenylate cyclase signaling by Rp-cAMPS triethylamine, we found no difference in the apoptotic cell-induced neutrophil chemoattractant formation between A3R or A2AR null macrophages and their wild-type counterparts indicating that the main receptor that triggers adenylate cyclase activity and inhibits neutrophil chemoattractant formation in the absence of A3R signaling must be the A2AR. This observation is further supported by the finding that inhibition of A2AR by signaling in A3R null engulfing cells results in similar amount of proinflammatory cytokine formation as the inhibition of the adenylate cyclase pathway by Rp-cAMPS. Our data also indicate that the main receptor that inhibits the adenylate cyclase pathway is the A3R. Previously we have shown that exogenously added NO alone is not sufficient to trigger neutrophil chemoattractant formation in macrophages, but enhances it, when it is administered to engulfing wild-type macrophages. This observation indicated that apoptotic cells induce neutrophil chemoattractant formation not only *via* inducing NO formation, but also simultaneously triggering additional signaling pathway(s). Since in A3R null engulfing macrophages, in which the adenylate signaling pathway is strongly activated, exogenous NO could enhance neutrophil chemoattractant formation only when it was added together with Rp-cAMPS triethylamine, our data indicate that the enhanced adenylate cyclase signaling must interfere with the additional pathway(s) as well. NO has been known to influence the expression of various pro-inflammatory cytokines for a long time, and it does so very likely by regulating the activity of various intracellular signaling molecules and transcription factors, which contain critical cysteine residues that undergo S-nitrosylation and denitrosylation. While low concentrations of it in mice, like in our case, stimulate MIP-2 and KC production, high concentrations were found to be anti-inflammatory [27]. Though it is a debate, human macrophages seem also to express inducible nitric oxide synthetase and can produce NO [28], but their NO production and the involvement of it in the regulation of proinflammatory cytokine formation during engulfment of apoptotic cells have not been studied yet. Altogether these observations indicate that apoptotic cells are capable of triggering proinflammatory responses in macrophages during engulfment, but these responses are counteracted by the adenosine produced. Our data demonstrate that in wild-type macrophages engulfing apoptotic cells initially both A2ARs and A3Rs are activated by adenosine, and a balance between the activities of the two receptors decides the strength of the adenylate cyclase signaling and the consequent degree of suppression of apoptotic cell-induced chemoattractant formation. But because A2ARs are upregulated, while A3Rs disappear from the cell surface of engulfing macrophages, adenosine on long term will mediate suppression of inflammation when macrophages engulf apoptotic cells.

Uncited reference

[16].

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References

[1] Savill J, Fadok V, Henson P, Haslett C. Phagocyte recognition of cells undergoing apoptosis. Immunol Today 1993;14:131–6.
[2] Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature 1997;390:350–1.
[3] Fadok VA, Bratton DL, Konowal A, Freed PW, Wescott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGFβ PGE_2 and PAF. J Clin Invest 1998;101:890–8.
[4] Cvetanovic M, Ucker DS. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled to specific recognition. J Immunol 2004;172:880–9.
[5] Cocco R, Ucker DS. Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. Mol Biol Cell 2001;12:919–30.
[6] Patel VA, Longacre-Antoni A, Cvetanovic M, Lee DJ, Feng L, Fan H, et al. The affirmative response of the innate immune system to apoptotic cells. Autoimmunity 2007;40:274–80.
[7] McDonald PP, Fadok VA, Bratton D, Hanson PM. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-β in macrophages that have ingested apoptotic cells. J Immunol 1999;163:6164–72.
[8] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:413–92.
[9] Fredholm BB. International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527–52.
[10] Haskó G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. Trends Immunol 2004;25:33–9.
[11] Köröskényi K, Duró E, Pallai A, Sarang Z, Kloor D, Ucker DS, et al. Involvement of adenosine A_{2A} receptors in engulfment-dependent apoptotic cell suppression of inflammation. J Immunol 2011;186:7144–55.
[12] Lee HT, Ota-Setlik A, Xu H, D'Agati VD, Jacobson MA, Emala CW. A3 adenosine receptor knockout mice are protected against ischemia- and myoglobinuria-induced renal failure. Am J Physiol Ren Physiol 2003;284:F267–73.
[13] Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ, et al. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. Nature 1997;388:674–8.
[14] Kleinbongard P, Rassaf T, Dejam A, Kerber S, Kelm M. Griess method for nitrite measurement of aqueous and protein-containing samples. Methods Enzymol 2002;359:158–68.
[15] Kronlage M, Song J, Sorokin L, Isfort K, Schwerdtle T, Leipziger J, et al. Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. Sci Signal 2010;3:ra55.
[16] Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, et al. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J Cell Biol 2001;155:649–59.
[17] Iida N, Grotendorst GR. Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. Mol Cell Biol 1990;10:5596–9.
[18] Wolpe SD, Sherry B, Juers D, Davatelis G, Yurt RW, Cerami A. Identification and characterization of macrophage inflammatory protein 2. Proc Natl Acad Sci USA 1989;86:612–6.
[19] Wolpe SD, Cerami A. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. FASEB J 1989;3:2565–73.
[20] Dinarello CA. The interleukin-1 family: 10 years of discovery. FASEB J 1994;8:1314–25.

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[21] van Haastert PJ, Van Driel R, Jastorff B, Baraniak B, Stec WJ, De Wit RJ. Competitive cAMP antagonists for cAMP-receptor proteins. J Biol Chem 1984;259:10020–4.
[22] Seamon KB, Padgett W, Daly JW. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci USA 1981;78:3363–7.
[23] Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol 2005;6:1191–7.
[24] Corriden R, Insel PA. New insights regarding the regulation of chemotaxis by nucleotides, adenosine, and their receptors. Purinergic Signal 2012;8: 587–98.
[25] Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. Cold Spring Harb Perspect Biol 2013;5:a008748.
[26] Hubbard NE, Lee S, Lim D, Erickson KL. Differential mRNA expression of prostaglandin receptor subtypes in macrophage activation. Prostaglandins Leukot Essent Fatty acids 2001;65:287–94.
[27] Kobayashi Y. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. J Leukocyte Biol 2010;88:1157–62.
[28] Kobzik L. Translating NO biology into clinical advances: still searching for the right dictionary. Am J Respir Cell Mol Biol 2009;41:9–13.

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