Involvement of Adenosine A_2A Receptors in Engagement-Dependent Apoptotic Cell Suppression of Inflammation

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Inflammation

Engulfment-Dependent Apoptotic Cell Suppression of Inflammation

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Efficient execution of apoptotic cell death followed by efficient clearance mediated by professional macrophages is a key mechanism in maintaining tissue homeostasis. Removal of apoptotic cells usually involves three central elements: 1) attraction of phagocytes via soluble “find me” signals, 2) recognition and phagocytosis via cell surface-presenting “eat me” signals, and 3) suppression or initiation of inflammatory responses depending on additional innate immune stimuli. Suppression of inflammation involves both direct inhibition of proinflammatory cytokine production and release of anti-inflammatory factors, which all contribute to the resolution of inflammation. In the current study, using wild-type and adenosine A2A receptor (A2AR) null mice, we investigated whether A2ARs, known to mediate anti-inflammatory signals in macrophages, participate in the apoptotic cell-mediated immunosuppression. We found that macrophages engulfing apoptotic cells release adenosine in sufficient amount to trigger A2ARs, and simultaneously increase the expression of A2ARs, as a result of possible activation of liver X receptor and peroxisome proliferators activated receptor &. In macrophages engulfing apoptotic cells, stimulation of A2ARs suppresses the NO-dependent formation of neutrophil migration factors, such as macrophage inflammatory protein-2, using the adenylate cyclase/protein kinase A pathway. As a result, loss of A2ARs results in elevated chemoattractant secretion. This was evident as pronounced neutrophil migration upon exposure of macrophages to an in vivo peritonitis model. Altogether, our data indicate that adenosine is one of the soluble mediators released by macrophages that mediate engulfment-dependent apoptotic cell suppression of inflammation. The Journal of Immunology, 2011, 186: 000–000.

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 (1). As they become apoptotic, cells undergo dramatic changes in the composition of their surface, which allows their recognition by phagocytes and subsequent removal.

Apoptotic cell clearance is believed to represent a critical process in tissue remodeling, maintenance of immune homeostasis, and resolution of inflammation (2, 3).

Whereas the phagocytosis of a variety of pathogenic targets, especially bacteria and virally infected cells, normally triggers a proinflammatory 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 simply fail to provide proinflammatory signals; rather, they actively interfere with the inflammatory program. For example, preincubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced via TLR4 by LPS, a component of the cell wall of Gram-negative bacteria (4–6). This inhibitory property appears to be a common attribute acquired posttranslationally by all cells undergoing apoptotic cell death, regardless of the cell type or the particular death stimulus (6–8).

The mechanism(s) by which apoptotic cells exert their inhibition on phagocytes may vary over time. The earliest anti-inflammatory activity of the apoptotic corpse is manifest as an immediate-early inhibition of macrophage proinflammatory cytokine gene transcription and is exerted directly upon binding to the macrophage, independent of subsequent engulfment and soluble factor involvement (6). This transcriptional inhibition is evident, for example, on the level of NF-κB-dependent transcription and occurs without effect on proximal signaling events induced by inflammatory receptors, including innate immune receptors of the TLR family (6). In case of IL-12, for example, cell–cell contact with apoptotic cells is sufficient to induce the inhibition of the
cytokine production by activated macrophages via a novel zinc finger NF, GC-BP, which selectively inhibits IL-12 p35 gene transcription (9).

Subsequently, soluble mediators may act in a paracrine or autocrine fashion to amplify and sustain the anti-inflammatory response. For example, Voll et al. (4) observed an inverse relationship between the secretion of the proinflammatory cytokines TNF-α, IL-1β, and IL-12 and the transient release of IL-10 from LPS-stimulated macrophages after their interaction with apoptotic cells. Fadok et al. (5) reported a similar inverse relationship between the release of TGF-β, platelet-activating factor, and PGE₂ and the secretion of TNF-α, IL-1β, and several other inflammatory cytokines by macrophages after engulfment of apoptotic targets. Although the pharmacologic blockade of platelet-activating factor and PGE₂ signaling had only slight effect (10), the addition of TGF-β-specific neutralizing Ab to cultures of macrophages and apoptotic targets partially restored proinflammatory cytokine release (5, 10) indicating a central role of this cytokine in mediating anti-inflammatory responses.

Adenosine is a purine nucleoside that, after its release from cells or after being formed extracellularly, diffuses to the cell membrane of surrounding cells, where it binds to its receptors (11, 12). There are four adenosine receptors, all of which are G protein-coupled receptors and are abundantly expressed by macrophages (13). The genes for these receptors have been analyzed in detail and are designated A₁, A₂A, A₂B, and A₃. Adenosine A₁ receptors are stimulated by 10⁻¹⁰ to 10⁻⁶ M concentrations of adenosine and mediate decreases in intracellular cAMP levels; adenosine A₂A receptors (A₂ARs) and A₂B receptors are stimulated by higher (5 × 10⁻⁷ M and 1 × 10⁻⁵ M, respectively) concentrations of adenosine and mediate increases in cAMP levels; and adenosine A₃ receptors are stimulated by 10⁻⁸ M concentrations of adenosine and mediate adenylyl cyclase inhibition (12). Although adenosine is constitutively present in the extracellular space at low concentrations (<1 μM), its concentration can dramatically increase under inflammatory conditions reaching concentrations high enough (14, 15) to exert immunomodulatory and especially immunosuppressive effects (13).

Because many of the immunosuppressive effects of adenosine on macrophages have been reported to be mediated via adenosine A₂ARs (13), we asked whether adenosine acts as a soluble mediator, via A₂ARs, to prevent proinflammatory cytokine production by macrophages engulfing apoptotic cells.

Materials and Methods
Reagents
All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except as indicated otherwise.

Animals
Most of the experiments were done using 3-mo-old male wild-type (WT) or A2AR-deficient mice (16) generated on an FVB background. Some of the experiments were also carried out on peroxisome proliferator-activated receptor α (PPARα)−/− (17) or liver X receptor (LXR)-null (18) mice. A2AR-deficient mice were generated on 129SvJ or on a mixed background of C57BL/6 and 129Sv, except as indicated otherwise.

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% CO₂ for 2 d before use. After 3–4 h incubation, the nonadherent cells were washed away. Before the experiments, the cells were cultured for 2 d replacing media daily. For bone marrow-derived macrophages, WT and PPARα null bone marrow was isolated from femurs and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% L929 conditioned media for 10 d. The nonadherent cells were washed away from the 3rd day medium.

Thymocyte apoptosis induction in vitro
Apoptotic cells were prepared from WT mice in all cases. Thymocytes isolated from 4-wk-old mice were cultured for 24 h (10⁷ cells/ml in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. In case of NB4 cells, the apoptosis was induced by 10 μM A2O₃ treatment for 12 h (19). This method typically resulted in >80% apoptotic cells (as assessed by propidium iodide/annexin V–FITC staining). Apoptotic cells were used at a 10:1 (apoptotic cell/macrophage) ratio.

Determination of the cell culture medium for adenosine measurement
The experiments were performed by coincubating A2AR+/+ macrophages (1 × 10⁶ cells/sample) with apoptotic cells in a 1:10 ratio. For the respective experiments, macrophages were pretreated with 50 nM cytochalasin D for 1 h to block the phagocytic activity of macrophages.

After 2 h of phagocytosis, the supernatants were replaced with fresh culture media. After 5 h of incubation (at 37°C), the supernatants were collected, deproteinized with 5 ml ice-cold 0.6 N HClO₄, and stored at −80°C. The determination of adenosine concentration was carried out with a reverse-phase HPLC method as described (20).

Determination of A2AR expression on the cell surface
WT and A2AR null peritoneal macrophages were coincubated with apoptotic thymocytes for 1 h in a 1:10 ratio. After replacing media and washing away the apoptotic cells, macrophages were incubated for an additional 1, 3, or 5 h. For some experiments, all these treatments were carried out in the presence of 5 μg/ml actinomycin D or 0.1 μg/ml cycloheximide (CHX). For characterizing the regulation of the expression of the receptor, macrophages were treated with various concentrations of 22-R(OH)-cholesterol, an LXR agonist, or GW501516, a PPARα agonist (GlaxoSmithKline) 2–3 h. After the treatments, macrophages were washed (1× PBS), blocked, with 50% FBS for 30 min, and labeled with anti-mouse A2AR Ab (BD Pharmingen) or goat IgG isotype control. For 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.

Determination of A2AR mRNA expression
WT, A2AR null, and LXR null peritoneal or PPARα WT and knockout bone marrow-derived 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) thymocytes for 1 h in a 1:10 ratio. After washing away the apoptotic cells and replacing media mRNA was collected 2 h later.

Determination of cytokine production
WT and A2AR null peritoneal macrophages were plated onto 24-well plates at a density of 5 × 10⁵ cells/well. To determine cytokine production by macrophages exposed to apoptotic cells, macrophages were exposed to apoptotic cells for 1 h in the presence or absence of an A2AR-selective antagonist, SCH442416 (10 nM; Torcix), or an A2AR agonist, 22-R(OH)-cholesterol (20 μM). Apoptotic cells then were washed away, SCH442416 or CHS21680 were re-added, 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; R&D Systems). The pixel density in each spot of the array was determined by ImageJ software. Alternatively, cytokine-induced neutrophil-attracting chemokine (KC), TGF-β, MIP-2, and IL-10 cytokine levels were measured with ELISA kits from R&D Systems.

To determine the effective amount of CGS21680 and SCH442416 for the cytokine array, WT peritoneal macrophages (5 × 10⁵ cells/sample) were treated with increasing amount of agonist or with increasing amount of antagonist in combination with 10 nM agonist. After 30-min pretreatment, MIP-2 production was triggered by the addition of 200 ng/ml bacterial LPS. 1-h incubation, LPS was washed away, the CGS21680 and SCH442416 were re-added, and the macrophages were cultured for an additional 5 h. The MIP-2 levels were determined by ELISA from cell culture media.
**Isolation of total cellular RNA and quantitative RT-PCR**

Total RNA was isolated from control and treated macrophages (3 × 10⁶ cells/sample) by TRI reagent. After various treatments, 3 × 10⁶ peritoneal macrophages were washed with ice-cold PBS. RNA was extracted with TRI reagent. cDNA was synthesized with High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s instructions. Cyclophilin, MIP-2, A2AR, NO synthase [inducible NO synthase (iNOS)] and endothelial NO synthase (eNOS), and arginine I and II levels were determined with TaqMan PCR using FAM–GMB–labeled primers (Applied Biosystems). Real-time PCR plates were run on an ABI Prism 7900 using ABM Prism SDS2.1 software for evaluation (Applied Biosystems). Samples were run in triplicate. Gene expression was normalized to cyclophilin expression.

**Phagocytosis assay**

For phagocytosis assays, macrophages were stained overnight with 10 μM CMTMR (Invitrogen), and thymocytes were labeled overnight with 6 μM CFDA (Invitrogen). Macrophages were incubated with apoptotic thymocytes in a 10:1 target/macrophage ratio for 1 h. Cells incubated with apoptotic thymocytes incubated at 4°C were used as controls. After washing, the cells on the plate were trypsinized, resuspended in cold medium with 0.5% sodium azide, and 10,000–20,000 cells were analyzed for each point by two-color flow cytometry (FACS CALIBUR; BD Biosciences).

For visualizing apoptotic cells in macrophages, macrophages were plated in 2-well chamber slides in a concentration of 5 × 10⁶/well and cultured for 48 h before CMTMR staining. 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.

**Determination of NO production of macrophages engulfing apoptotic cells**

A2AR+/+ or A2AR−/− 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–Flossovay method (21).

**In vivo neutrophil migration assay**

A2AR+/+ and A2AR−/− mice were injected with 2 ml 4% thioglycolate i.p. Four days later, they were injected i.p. with 2 × 10⁶ apoptotic cells suspended in 2 ml physiological saline or with 2 ml physiological saline alone. After 3 h, the peritoneal cells were collected, washed, blocked with 50% FBS, and stained with FITC-conjugated rat anti-mouse Gr-1 (RB6-8C5; Pharmingen) or V450 rat IgG2b isotype control for 30 min. Detection was carried out using FITC-conjugated anti-rat IgG (Pharmingen). Cells were then washed, fixed, and analyzed by flow cytometry (FACS CALIBUR; BD Biosciences) to determine the percentages of neutrophils in the total cell population. In some experiments, rat anti-mouse KC (clone 48415.111; IgG2a), rat anti-mouse MIP-2 (clone 40605; IgG2b), or their isotype controls IgG2a (clone 5444.11) and IgG2b (clone 141945) obtained from R&D Systems were injected together with the apoptotic cells into A2AR−/− mice.

**Statistical analyses**

All data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± SD. The p values were calculated by using two-tailed Student t test for two samples of unequal variance. The analysis of cytokine array experiments was carried out by ANOVA test. A p value <0.05 was considered to be statistically significant.

**Results**

Adenosine is produced and the expression of A2ARs is enhanced on the cell surface of macrophages during engulfment of apoptotic cells

We first tested whether macrophages engulfing apoptotic cells for 2 h release adenosine during the following 5 h. Whereas the adenosine levels in the culture medium alone or the culture medium of macrophages or thymocytes cultured alone for the same time period were below the detection limit (0.1–0.25 μM), adenosine was found in the culture medium of macrophages exposed previously to apoptotic cells (3.34 ± 1.4 μM, n = 3 independent experiments). Induction of the release of adenosine was not specific for thymocytes, as other apoptotic targets, such as uptake of As2O₃-treated apoptotic NB4 acute promyelocytic leukemia cells (19), also resulted in adenosine release (67.49 ± 6.32 μM, n = 3). This concentration of adenosine is in the range of the A2AR sensitivity. Inhibition of phagocytosis by cytchalasin D, an inhibitor of actin polymerization, completely prevented the appearance of adenosine, indicating that the production is dependent on apoptotic cell engulfment.

As shown in Fig. 1A, mouse peritoneal macrophages engulfing apoptotic cells not only produce adenosine but also express A2ARs, and this expression is significantly enhanced after incubation with apoptotic cells. Induction in A2AR expression during phagocytosis was specific for the engulfment of apoptotic cells, as uptake of neither necrotic nor Ab-coated cells affected it (Supplemental Fig. 1). Preincubation of macrophages for 30 min with actinomycin D, a transcription inhibitor, or CHX, a protein synthesis inhibitor, prevented the apoptotic cell–associated induction of adenosine receptor expression, indicating that regulation occurs on the transcriptional level (Fig. 1B). Indeed, induction on the level of mRNA was evident after the engulfment of apoptotic cells (Fig. 1C).

Cytochalasin D does inhibit the engulfment process, but it does not influence the recognition of apoptotic cells (6). Binding of phosphatidylserine on the surface of apoptotic cells plays a 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 (22)]. Both cytchalasin D and recombinant annexin V inhibited the induction of A2AR expression by apoptotic cells (Fig. 1C, 1D) suggesting that it is the engulfment of apoptotic cells, rather than their recognition per se, that triggers enhanced A2AR expression.

Recently, two lipid-sensing nuclear receptors (LXR and PPARδ) expressed by macrophages have been implicated in promoting phagocytosis and anti-inflammatory effects of apoptotic cells after their uptake (17, 18). Both 22-(R)-OH-cholesterol, an LXR agonist, and GW501516, a PPARδ agonist, promoted the mRNA expression of A2AR in peritoneal macrophages (Fig. 1E, 1F) indicating that both LXR and PPARδ might mediate the effect of apoptotic cell engulfment on A2AR expression. Because the effect of these agonists might not be fully specific, LXR knockout and PPARδ knockout macrophages were also tested for their response to prove further the involvement of these receptors in A2AR up-regulation. Whereas in case of PPARδ knockout macrophages the up-regulation of A2AR was attenuated compared with the WT control (Fig. 1G), we could not draw a definite conclusion from the LXR knockout mice, as the WT control did not show an up-regulation (Fig. 1H). We have no explanation why induction of the expression of A2AR was seen in macrophages on FVB and 129/Sv backgrounds but was not seen in mice on a mixed background of C57BL/6 and 129Sv. These data imply that lipid-sensing receptors might mediate the effect of apoptotic cells on the A2ARs, but as the biological activity of these receptors overlaps, only testing double-knockout cells would give a full answer.

Loss of A2AR influences the proinflammatory cytokine production by macrophages engulfing apoptotic cells

Evaluation of the cytokine secretion profile of unstimulated macrophages was performed using a highly sensitive cytokine Ab 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 WT and A2AR−/− macrophages in vitro. The results reported in Fig. 2B show that ∼85% of all available cytokines on the filters were detectable, even though some were at very low levels. The loss of the A2AR did not affect significantly the level or the composition of most of the cytokines released. However, as shown in Fig. 2C, when macrophages were exposed to apoptotic cells, we found nine cytokines whose production, although not affected in WT cells, was increased in A2AR−/− macrophages.

FIGURE 1. Apoptotic cell uptake leads to de novo A2AR synthesis in peritoneal macrophages. A and B, WT peritoneal macrophages were exposed for 1 h to apoptotic thymocytes alone (A) or in combination with 0.1 μg/ml CHX or 10 μM actinomycin D (B). Thymocytes were then washed away, and macrophages were further incubated for the indicated time periods (A) or for 3 h (B). Cell surface expression of A2AR was determined by flow cytometry. C and D, Blocking of apoptotic cell phagocytosis abolishes the increase of A2AR 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/10⁵ apoptotic cells). After 1-h coinubcation, apoptotic cells were washed away, and macrophages were further cultured for 2 h (C) or 3 h (D). A2AR level was determined by quantitative PCR (C) or by flow cytometry (D). E and F, Macrophages were treated with GW501516, a PPARδ agonist (E), or with 22-(R)OH-cholesterol, an LXR agonist (F), for 3 h. Cell surface expression of A2AR was determined by flow cytometry. G and H, A2AR gene expression level was also determined in PPARδ knockout bone marrow-derived macrophages (G) and LXR null peritoneal macrophages (H). Macrophages were coincubated with apoptotic thymocytes for 1 h. Apoptotic cells were then washed away, and macrophages were further incubated for 2 h. The gene expression levels were measured by quantitative PCR. Results are expressed as mean ± SD of three or four independent experiments. *p < 0.05. MFI, mean fluorescence intensity.
These cytokines include the IFN-γ-inducible protein 10 kDa, KC, and MIP-1α, -1β, and -2, which act as chemoattractants for neutrophils and/or other cell types (23–26). The proinflammatory cytokines IL-17 (27) and IL-1α (28), as well as IL-3, which stimulates the differentiation of multipotent hematopoietic stem cells into the myeloid direction and proliferation of all cells in the myeloid lineage (29), also were produced in an enhanced amount. In addition, release of the antagonist of the IL-1 receptor (IL-1ra) was also enhanced. The induction or the modification in the levels of these cytokines was not due to the presence of high amount of late apoptotic cells or altered phagocytosis, as the majority of apoptotic cells were propidium iodide-negative (Fig. 2D), and no differences in the extent of phagocytosis of WT and A2AR−/− macrophages were observed (Fig. 2E–G). Among the cytokines released in altered amounts in the supernatant by cultured A2AR−/− 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,C). No cytokines were detected in the supernatants when thymocytes (viable or apoptotic) were incubated alone (data not shown), demonstrating

FIGURE 2. A2AR-deficient macrophages respond to apoptotic cells with enhanced 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 WT and A2AR null peritoneal macrophages. Peritoneal macrophages were coincubated 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 neutrophil chemoattractants, which are significantly overproduced by A2AR null macrophages. C, Cytokines, the levels of which were significantly different (p < 0.05) in the supernatants analyzed by cytokine array. The density of MIP-1α and -1β was low to display on the same scale together with the other cytokines. D, Flow cytometric analysis of annexin V–FITC- and propidium iodide-stained apoptotic thymocytes. E, Flow cytometric measurement of capacity of WT and A2AR null peritoneal macrophages to take up apoptotic thymocytes. F and G, The number of engulfed CFDA labeled apoptotic cells within CMTMR labeled WT or A2AR-null macrophages counted by confocal microscopy after 1 h of phagocytosis. Original magnification ×400. Results are expressed as mean ± SD of three independent experiments.

s.b., surface-bound cells.
that the secreted cytokines were macrophage related. To make sure that alterations in the cytokine profile were not a result of adenosine receptor-related but developmental effects in the A2AR knockout versus WT macrophages, cytokine release of macrophages exposed to apoptotic cells was also tested in the presence of the highly specific A2AR antagonist SCH442416 and the highly specific A2AR agonist CGS21680. The way that the selective concentration of these compounds was determined is described in Supplemental Fig. 2. As shown in Fig. 3A–C, using WT macrophages we could confirm the enhanced expression of KC and MIP-2 in the presence of an A2AR antagonist as well, indicating that the altered pattern of KC and MIP-2 secretion observed in A2AR−/− macrophages is indeed a consequence of the lack of A2AR signaling during phagocytosis of apoptotic cells. In addition, the enhanced expression of MIP-1α and MIP-1β, two additional neutrophil chemotactants (26), was more clearly seen. Administration of CGS21680, in contrast, further decreased the amount of KC and MIP-2 produced by WT macrophages engulfing apoptotic cells but had no significant effect on the amount of KC and MIP-2 produced by A2AR null macrophages engulfing apoptotic cells (Fig. 3D–F). To confirm the results, MIP-2 and KC protein levels also were assessed by ELISA. In harmony with the cytokine array results, although the production of MIP-2 and KC are not altered by WT macrophages exposed to apoptotic cells, the release of both MIP-2 and KC by A2AR−/− macrophages is enhanced under the same conditions (Fig. 4A, 4B).

**Macrophages not expressing A2ARs induce migration of neutrophils when exposed to apoptotic cells in a sterile peritonitis model**

Because we found that in the absence of A2ARs, macrophages release the neutrophil chemoattractant MIP-2 (CXCL-2) and KC (CXCL-1) when they engulf apoptotic cells, we decided to investigate whether in A2AR−/− mice injection of apoptotic cells affects migration of neutrophils in a sterile peritonitis model. For this purpose, mice were injected i.p., first with thioglycolate and then, 4 d later, with $2 \times 10^6$ apoptotic cells. As shown in Fig. 5A, injection of apoptotic thymocytes did not induce a significant neutrophil migration into the peritoneum in WT mice, whereas in the A2AR−/− mice, a significant neutrophil migration was detected in this model. This was accompanied by enhanced levels of MIP-2 and KC in the peritoneal fluid of A2AR−/− mice (Fig. 5B). To test whether the enhanced production of KC and MIP-2 together are responsible for the phenomenon, blocking Abs anti–KC (50 μg) and anti–MIP-2 (50 μg) or their isotype controls were added together with the apoptotic cells. As shown in Fig. 5A, addition of blocking Abs to both KC and MIP-2 completely prevented the migration of neutrophils, whereas their isotype controls

![FIGURE 3.](image-url) Compared with WT controls, MIP-2 and KC production is enhanced by both A2AR null or A2AR antagonist-treated WT macrophages engulfing apoptotic cells but decreased in WT A2AR agonist treated, apoptotic cell engulfing macrophages. A, Cytokine panel of control, apoptotic cell exposed, and SCH442416 (A2AR-specific antagonist) treated WT macrophages. Peritoneal macrophages were preincubated with 10 nM SCH442416 for 30 min, then they were exposed to apoptotic thymocytes for 1 h (MPh/APO = 1:10). Five hours after the removal of apoptotic cells, supernatants were collected, and cytokine levels were determined by cytokine array. Arrows in A highlight KC (B), MIP-2 (C), and MIP-1α and -1β overproduced by SCH442416-treated macrophages. D, Cytokine panel of control, apoptotic cell exposed, and CGS21680 (A2AR-specific agonist) treated WT and A2AR null macrophages. Peritoneal macrophages were preincubated with 10 nM CGS21680 for 30 min, then they were exposed to apoptotic thymocytes for 1 h (MPh/APO = 1:10). Five hours after the removal of apoptotic cells, supernatants were collected, and cytokine levels were determined by cytokine array. Arrows in D highlight MIP-2 (E) and KC (F) downregulated by CGS21680 in WT but not in A2AR null macrophages. Results are expressed as mean ± SD of three independent experiments. *p < 0.05.
had no effect. Addition of blocking Abs to MIP-2 alone did not fully block the migration of neutrophils. These data indicate that the loss of A2ARs leads to sufficient neutrophil chemoattractant production by macrophages engulfing apoptotic cells to affect migration of neutrophils in an in vivo peritonitis model.

Production of MIP-2 by A2AR−/− macrophages exposed to apoptotic cells is related to lack of protein kinase A-mediated inhibition of NO production

Because among the neutrophil chemoattractants, the release of which was altered by the loss of A2AR, MIP-2 showed the most dramatic changes during phagocytosis of apoptotic cells, we decided to investigate further the alterations in the regulation of this cytokine. Because previous studies indicated that in long-term (1 d) experiments TGF-β1 and IL-10 might mediate the anti-inflammatory effects of apoptotic cells (4, 5, 10), we checked whether TGF-β1 or IL-10 release is altered in macrophages lacking A2AR. However, in such short-term experiments we could not detect the release of IL-10 either with the cytokine array or by the ELISA technique. Active TGF-β was detectable, but its production was not altered in the A2AR−/− macrophages (Fig. 4C).

These data indicate that an altered IL-10 of TGF-β production is

FIGURE 4. Cytokine production of WT and A2AR null mice during phagocytosis of apoptotic cells. WT and A2AR null macrophages were exposed to apoptotic thymocytes for 1 h (MPh/APO = 1:10). Five hours after the removal of apoptotic cells, supernatants were collected. The levels of (A) MIP-2, (B) KC, and (C) active TGF-β production were determined by ELISA. Results are expressed as mean ± SD of three independent experiments. *p < 0.05.

FIGURE 5. Increased MIP-2 production is accompanied by enhanced neutrophil migration in vivo. A, WT and A2AR null mice with sterile peritonitis were i.p. injected with apoptotic cells [10^5 cells/mouse in physiological saline (apo. cell)] or vehicle alone. Untreated control mice were uninjected. In some cases, neutralizing MIP-2 and neutralizing KC Abs (nMIP-2 and nKC Abs) or their isotype controls were also injected. Three hours later, peritoneal cells were collected and analyzed by flow cytometry for Gr-1 positivity. B, The levels of MIP-2 and KC in the lavage fluids were determined by ELISA at the same time point. Results are expressed as mean ± SD of five independent experiments. *p < 0.05.
not responsible for the altered MIP-2 production in A2AR−/− macrophages. That is why we decided to investigate a possible direct regulation by the A2ARs. First, we tested the role of the adenylate cyclase pathway, as many of the anti-inflammatory effects of the A2AR were reported to be mediated via this signaling pathway (13). For this purpose, we exposed A2AR−/− macrophages to various compounds known to elevate intracellular cAMP levels: cholera toxin, which is known to increase cAMP levels by ADP-ribosylation of the stimulatory G protein of adenylate cyclase (30); forskolin, an adenylate cyclase activator (31); and dibutyryl-cAMP, a membrane-permeable variant of cAMP. Preincubation of A2AR−/− macrophages with these compounds for 30 min prevented the increase in MIP-2 levels when exposed to apoptotic cells (Fig. 6A). In contrast, preincubation of WT macrophages with Rp-cAMP triethylamine, a specific membrane-permeable inhibitor of cAMP-dependent protein kinase I and II (32), or recombinant adenosine deaminase, which degrades adenosine, resulted in an increase in MIP-2 production when exposed to apoptotic cells (Fig. 6B). These data together indicate that MIP-2 production by macrophages exposed to apoptotic cells is actively suppressed by the A2AR stimulated by adenosine in an autocrine way using the adenylate cyclase/protein kinase A signaling pathway.

Preincubation of A2AR−/− macrophages for 30 min with actinomycin D, a transcription inhibitor, or CHX, a protein synthesis inhibitor, prevented the apoptotic cell-associated induction of MIP-2 release, indicating that regulation occurs at the transcriptional level (Fig. 6C). Indeed, engulfment of apoptotic cells induced the mRNA levels of MIP-2 in A2AR−/− macrophages but not in their WT counterparts (Fig. 6D).

Previous studies have shown that NO is released by macrophages exposed to early apoptotic cells (33) and that NO can contribute to MIP-2 production (34, 35). To investigate the potential role of NO in the apoptotic cell-induced MIP-2 production in A2AR−/− macrophages, macrophages were exposed to the NO synthase (NOS) inhibitor L-NAME before the addition of apoptotic cells. Addition of L-NAME attenuated MIP-2 protein (Fig. 7A) and mRNA (Fig. 7B) expression in both WT and A2AR−/− macrophages induced by exposure to apoptotic cells, indicating that NO production contributes to the effect. We further investigated whether NO production is altered in the absence of A2AR. As shown in Fig. 7C, in accordance with previous reports (34), macrophages exposed to apoptotic cells produce NO. Indeed, apoptotic cell-induced NO production was enhanced in A2AR−/− macrophages compared with their WT counterparts. Because the addition of sodium nitroprusside, a potent NO donor, enhanced apoptotic cell-induced MIP-2 production in WT macrophages (Fig. 7D), our data indicate that NO contributes to the apoptotic cell-induced MIP-2 production. However, addition of sodium nitroprusside to macrophages alone was not able to induce MIP-2 production, implying that apoptotic cell-derived signals contribute to the induction of MIP-2 (Fig. 7D).

Similar to the induction of MIP-2 (Fig. 6A, 6B), production of NO by A2AR−/− macrophages engulfing apoptotic cells was inhibited by the adenylate cyclase activator forskolin (Fig. 7E), whereas it was enhanced in WT macrophages by the protein

**FIGURE 6.** Transcriptionally induced MIP-2 production by macrophages exposed to apoptotic cells is actively suppressed by the A2AR stimulated by adenosine in an autocrine way using the adenylate cyclase/protein kinase A signaling pathway. A, A2AR null peritoneal macrophages were exposed to apoptotic cells alone or after a 30-min pretreatment with forskolin (10 μM), cholera toxin (100 ng/ml), or dibutylryl-cAMP (100 μM). B, WT peritoneal macrophages were exposed to apoptotic cells alone or in combination with 100 μM Rp-cAMP triethylamine or adenosine deaminase (1 U/ml). C, WT or A2AR null peritoneal macrophages were exposed to apoptotic cells alone or after a 30-min pretreatment with 5 μg/ml actinomycin D or 0.1 μg/ml CHX. In all these experiments (A–C), apoptotic cells were washed away after 1 h, and supernatants were collected after 5 h to determine MIP-2 production by ELISA. D, Quantitative RT-PCR analysis of MIP-2 mRNA expression in WT resting, 1-h apoptotic cell-exposed peritoneal macrophages at 2 h after the removal of the apoptotic cells or apoptotic thymocytes. Expression values are represented as mean amount of target mRNA normalized to the expression of cyclophilin. Results are expressed as mean ± SD of five independent experiments. *p < 0.05.
kinase A inhibitor Rp-cAMP triethylamine (Fig. 7F) suggesting that A2AR-mediated adenylate cyclase signaling inhibits primarily NO production.

Enhanced NO production in A2AR−/− macrophages is accompanied by altered expressions of iNOS and arginase II

NO can be produced by three NO synthase isoenzymes, two of which, eNOS and iNOS, are found in macrophages. eNOS is expressed constitutively, and its activity is dynamically regulated by Ca2+-calmodulin. iNOS is not expressed normally and is highly inducible. The capacity of NOS to synthesize NO also is determined by the amount of its substrate, arginine, the level of which is negatively regulated by arginases (36). We investigated the expression of various genes encoding enzymes related to NO production, such as arginase I and II, iNOS, and eNOS. eNOS was not detectable in resting or engulfing macrophages, suggesting that iNOS is responsible for the apoptotic cell-associated NO production. In line with the higher NO production, the expression of iNOS was significantly higher in A2AR−/− macrophages than in their WT counterparts (Fig. 8A). Exposure to apoptotic cells did not alter the levels of arginase I but induced a downregulation in iNOS and an upregulation in the arginase II expression, favoring the use of arginine in the production of polyamines. However, both the upregulation of arginase II and the downregulation of iNOS by apoptotic cells were delayed in A2AR−/− macrophages (Fig. 8B). All these data indicate that exposure to apoptotic cells

FIGURE 7. Apoptotic cell-induced MIP-2 production by A2AR null macrophages requires NO production. A and B, L-NAME, a NOS inhibitor, prevents apoptotic cell-induced MIP-2 production by macrophages on both protein (A) and mRNA (B) levels in a dose-dependent manner. WT or A2AR null peritoneal macrophages were exposed to apoptotic cells for 1 h alone or after a 30-min pretreatment with L-NAME at the indicated concentrations. After 1 h, apoptotic cells were washed away, and mRNA levels were determined 2 h later and cytokine levels in the supernatant 5 h later, L-NAME being constantly present. mRNA levels are expressed as fold changes compared with the resting macrophages. C, NO production of macrophages exposed to apoptotic cells is enhanced in the absence of A2AR. Macrophages were exposed to apoptotic cells for 1 h. NO production was determined after an additional hour. D, The NO donor sodium nitroprusside enhances MIP-2 production, but only in macrophages exposed to apoptotic cells. WT peritoneal macrophages were exposed to the indicated concentrations of sodium nitroprusside in the presence or absence of apoptotic cells. Apoptotic cells were washed away after 1 h. MIP-2 mRNA levels were determined 2 h later. E, Forskolin, an adenylate cyclase activator, decreases NO production by A2AR−/− macrophages. A2AR−/− peritoneal macrophages were exposed to apoptotic cells for 1 h alone or after a 30-min pretreatment with forskolin (10 μM). Apoptotic cells were washed away, and NO production was determined after an additional hour. F, Inhibition of protein kinase A enhances NO production of WT macrophages exposed to apoptotic cells. WT peritoneal macrophages were exposed to apoptotic cells for 1 h alone or after a 30-min pretreatment with 100 μM Rp-cAMP triethylamine. Apoptotic cells were washed away, and NO production was determined after an additional hour. Results are expressed as mean ± SD of five independent experiments. *p < 0.05.
induces a modification in the arginine metabolism that favors polyamine production. But this shift is delayed in A2AR null macrophages.

Because the A2AR-induced adenylate cyclase pathway suppresses NO production, we tested whether influencing the adenylylate cyclase pathway alters arginase II or iNOS expression. As shown in Fig. 8C, inhibition of protein kinase A by Rp-cAMP triethylamine in WT macrophages enhances the expression of iNOS, whereas the addition of forskolin to A2AR null macrophages inhibits it (Fig. 8D). Similar manipulations altered the expression of arginase II conversely.

Discussion

Acute inflammation normally resolves by mechanisms that are initiated in the first few hours after an inflammatory response begins. The resolution program involves phagocytosis of apoptotic neutrophils by macrophages, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as TGF-β1 (37).

The current study investigated whether adenosine and one of its receptors, the A2AR, could be involved in the anti-inflammatory response induced in macrophages by apoptotic cells. We have shown that macrophages engulfing apoptotic cells produce adenosine at levels that can trigger A2ARs and, at the same time, elevate the expression of the receptor itself. Thus, adenosine can act in an autocrine manner during phagocytosis. Loss of A2ARs did not affect the rate of phagocytosis. This was a surprise for us, as increases in cAMP levels were reported to inhibit engulfment of apoptotic cells (38). However, when exposed to apoptotic cells, A2AR−/− macrophages notably produced increased amounts of MIP-2 and KC acting as chemoattractants for various cell types, especially for neutrophils. We could confirm these data using a specific A2AR agonist and an A2AR antagonist indicating that lack of actual A2AR signaling rather than altered macrophage differentiation in the absence of A2AR explains the phenomenon.

These data suggest that during engulfment of apoptotic cells, especially when macrophages participate in the resolution of inflammation, where they clear large numbers of apoptotic neutrophils (39), A2ARs might participate in the negative feedback control of neutrophil transmigration to the inflammation site. Because inflammatory cytokines were shown to sensitize A2ARs (40), the role of adenosine mediating the inhibitory effect of apoptotic cells might be more significant under inflammatory conditions than was observed in our in vitro model, which lacked inflammation. In support of this hypothesis, enhanced production of MIP-2 and KC by A2AR−/− macrophages engulfing apoptotic cells was shown in an in vivo peritonitis model, and this was accompanied by MIP-2− and KC-dependent neutrophil migration, which was not seen in WT mice.

In our further experiments, MIP-2 production by A2AR−/− macrophages was studied in detail. Although previous studies have shown that apoptotic cell-induced IL-10 production in macrophages can negatively regulate the production of proinflammatory cytokines (4), and A2ARs were reported in certain inflammatory contexts to promote IL-10 formation (41), we found no detectable IL-10 production in our experimental system. Instead, we found that MIP-2 synthesis was partially related to an enhanced NO production by A2AR−/− macrophages engulfing apoptotic cells that regulated MIP-2 production on a transcriptional level. Enhanced NO production of A2AR null macrophages compared with that of the wild types seems to be related to higher levels of iNOS, which produces NO, and lower levels of arginase II, which normally degrades arginine, the substrate of NO synthesis, prior to and after apoptotic cell exposure. However, mRNA levels alone might not reflect the real activities or activity ratio of these enzymes, as just iNOS activity alone was shown to be regulated by various signals on transcriptional, mRNA, translational,
and posttranslational levels (42, 43). In accordance with the existence of these additional regulation levels, we observed that although iNOS is already expressed on the mRNA level by resting macrophages, NO production is seen only after the apoptotic cell uptake indicating that apoptotic cells must induce an early signaling pathway that triggers NO production. Our attempts to identify this signaling pathway have failed to date. Because both WT and A2AR null cells produce NO after engulfment, our current hypothesis is that this pathway might not be affected by A2ARs. Instead, the inverse levels of iNOS and the arginase II affected by the A2ARs will determine the magnitude of the NO response. In support of our hypothesis, alterations in the arginine metabolism (favoring the arginase pathway leading to polyamine synthesis and inhibiting the synthesis of NO) after engulfment of apoptotic cells have already been reported (44). Notably, both TGF-β released by macrophages engulfing apoptotic cells (42, 45) and compounds known to activate protein kinase A (46, 47) were shown to increase arginase activity and decrease NO production in macrophages indicating that both TGF-β and A2ARs, which activate protein kinase A, might mediate or support the effect of apoptotic cells on the arginine metabolism in macrophages engulfing apoptotic cells. The role of TGF-β was proved previously (44), and our data indicate the additional involvement of A2ARs. Together, our data demonstrate that, besides TGF-β and IL-10 (4, 5), adenosine also participates in the negative regulation of proinflammatory cytokine production of macrophages engulfing apoptotic cells. In this context, adenosine uses the A2AR pathway and inhibits primarily neutrophil chemotrafficking formation and the consequent neutrophil immigration.

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Disclosures

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SUPPL. FIGURE 1.

![Bar graph showing ADORA2 fold change relative to gene expression across different conditions: apoptotic Tc, viable Tc, anti-CD3 Tc, heat killed Tc.](image)

- Bar graph illustrates the fold change in ADORA2 gene expression for various treatments.
- The x-axis represents different conditions, and the y-axis shows the fold change.
- The graph indicates a significant increase in ADORA2 expression (+) compared to controls (-).
- The asterisk (*) denotes statistical significance.
SUPPL. FIGURE 2.

A

B

MIP-2

inhibition %

LPS

+ 

+ 

+ 

100

1000

CGS21680 (nM)

A2AR^{+/+}

A2AR^{-/-}

blocking % of GS21680 effect

LPS

+ 

+ 

+ 

+ 

CGS21680 (10 nM)

SCH442416 (nM)

0.5

1.0

5.0

10.0
Supplementary Figure 1. The expression of adenosine A2A receptors is induced only following engulfment of apoptotic cells. Wild type peritoneal macrophages were co-incubated with various target cell types: apoptotic, living, heat killed (45 min, 55°C) or anti-CD3-pretreated (10 μg/ml, 20 min) thymocytes for 1 hr in 1:10 macrophage/target cell ratio. After washing away the target cells and replacing media, mRNA was collected 2 hours later. Results are expressed as mean ± S.D. of four independent experiments. (*p<0.05).

Supplementary Figure 2. Determination of the selective concentration of CGS21680, an A2AR agonist, and SCH442416, an A2AR antagonist. Wild-type and A2AR null peritoneal macrophages were pretreated with increasing amount of (A) CGS21680 or (B) that of SCH442416 in combination with 10 nM CGS21680 for 30 min. Then MIP-2 production was induced by the addition of LPS (200ng/ml). After 1 hr incubation LPS was washed away, but CGS21680 and SCH442416 were re-added and the macrophages were cultured in fresh media for an additional five hrs., at the end of which MIP-2 levels were determined by ELISA. LPS-induced MIP-2 levels were 2723.72±680.45 pg/ml in the wild-type samples and 4657.44±716.45 pg/ml in the A2AR null ones. In the case of agonist (A) the percentages of inhibition were calculated by comparing the MIP-2 levels in the agonist treated samples to the LPS control (LPS control= 0% inhibition). In the case of the antagonist (B), the inhibitory effect of the agonist CGS21680 (10 nM) (0% inhibition) was prevented by increasing amount of antagonist. Results are expressed as mean ± S.D. of three independent experiments.