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Human Eosinophils Recognize Endogenous Danger Signal Crystalline Uric Acid and Produce Proinflammatory Cytokines Mediated by Autocrine ATP

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Eosinophils are multifunctional leukocytes involved in various inflammatory processes, as well as tissue remodeling and immunoregulation. During inflammation and infection, injured cells and damaged tissues release uric acid and monosodium urate (MSU) crystals as important endogenous danger signals. Uric acid is also implicated in the immunogenic effects of an authentic Th2 adjuvant, aluminum hydroxide. Eosinophils often localize at sites of Th2-type chronic inflammation; therefore, we hypothesized that eosinophils may react to endogenous danger signals. We found that human eosinophils migrate toward soluble uric acid and MSU crystals in a gradient-dependent manner. Eosinophils incubated with MSU crystals, but not those incubated with uric acid solution, produced elevated levels of IL-6 and IL-8/CXCL8. Other cytokines and chemokines, including IL-1β, IL-10, IL-17, IFN-γ, CCL2, CCL3, CCL4, TNF-α, G-CSF, GM-CSF, fibroblast growth factor, vascular endothelial growth factor, and TGF-β, were also produced by eosinophils incubated with MSU crystals. Eosinophils exposed to MSU crystals rapidly (i.e., within 1 min of exposure) released ATP into the extracellular milieu. Importantly, this autocrine ATP was necessary for eosinophils to produce cytokines in response to MSU crystals, and P2 nucleotide receptors, in particular $P2Y_2$, are likely involved in this positive feedback loop. Finally, at higher concentrations, MSU crystals promoted P2R-dependent release of a granule protein (eosinophil-derived neurotoxin) and cell death. Thus, human eosinophils may respond to particulate damage-associated endogenous danger signals. These responses by eosinophils to tissue damage may explain the self-perpetuating nature of chronic inflammation in certain human diseases, such as asthma. ***The Journal of Immunology*****, 2010, 184: 6350–6358.**

Historically, eosinophils have been considered effector cells involved in host protection against helminth infections and in pathological processes in bronchial asthma and allergic diseases (1, 2). Activated eosinophils release toxic granule proteins and proinflammatory mediators, which may cause tissue damage and dysfunction (3). Recent evidence suggests that eosinophils may also be involved in tissue remodeling and immunoregulation (4). Eosinophils can synthesize, store, and secrete ≥35 inflammatory and immunoregulatory cytokines, chemokines, and growth factors (5), which may play roles in the regulatory functions of eosinophils. In several eosinophil-associated human diseases (e.g., asthma), eosinophil-derived TGF-β is linked with tissue remodeling (6). In eosinophil-deficient mice, T cell recruitment is impaired, and Th2 cytokine production is reduced (7–9). However, the cellular and molecular mechanisms involved for eosinophils to produce these tissue-remodeling and immunoregulatory cytokines are mostly unknown.

Inflammation and immune responses can be considered reactions to noxious stimuli and conditions, such as infection and injury (10). On one hand, infection with microorganisms, which express pathogen-associated molecular pattern molecules, is an example of an exogenous inducer of inflammation. Human eosinophils express TLRs (e.g., TLR7) (11), β2 integrins (12), and protease-activated receptors (13), which may recognize infectious agents. On the other hand, during tissue injury, endogenous molecules released by stressed or damaged tissues, such as ATP, K^+ ions, uric acid, HMGB-1, and S100 calcium-binding protein family members (14–16), may trigger inflammation. Indeed, the immunological actions of a prototypic Th2-type adjuvant (i.e., aluminum hydroxide) are likely mediated by the induction of endogenous uric acid (17). These damage-associated molecular pattern (DAMP) molecules or danger signals may play important roles in asthma and allergic disorders (18). However, whether and how eosinophils respond to DAMPs or danger signals is not clear.

In this article, we describe the eosinophils' attraction to a prototypic danger signal, namely monosodium urate (MSU) crystals (19). Eosinophils cultured with MSU crystals produce large quantities and various kinds of cytokines and chemokines. Importantly, the autocrine release of ATP likely provides a pivotal positive feedback signal in eosinophils exposed to MSU crystals. Thus, human eosinophils may respond to certain DAMPs released by injured cells or damaged tissues. This previously unrecognized capacity of eosinophils to respond to danger signals may explain the self-perpetuating nature of chronic inflammation in certain human diseases, such as asthma.

Materials and Methods

Reagents

Recombinant human IL-33 and human eotaxin were from R&D Systems (Minneapolis, MN). Recombinant human IL-5 was a generous gift from the Schering-Plough Research Institute (Kenilworth, NJ). Anti–CD16-conjugated

Division of Allergic Diseases, Department of Medicine and Department of Immunology, Mayo Clinic, Rochester, MN 55905

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Address correspondence and reprint requests to Dr. Hirohito Kita, Department of Medicine and Department of Immunology, Mayo Clinic Rochester, 200 First Street SW, Guggenheim Building, Room 401, Rochester, MN 55905. E-mail address: kita.hirohito@mayo.edu

Abbreviations used in this paper: ATPγS, ATP tetralithium salt; DAMP, damage-associated molecular pattern; EDN, eosinophil-derived neurotoxin; MFI, mean fluorescence intensity; MSU, monosodium urate; oATP, ATP periodate oxidized sodium salt; PAB, PBS with 3% BSA and 0.1% sodium azide; PI, propidium iodide; UA, uric acid.

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immunomagnetic beads and anti–FITC-conjugated immunomagnetic beads were from Miltenyi Biotec (Auburn, CA). FITC-conjugated anti-human CD14 mAb was from BD Biosciences (San Jose, CA). Percoll was from GE Healthcare Biosciences (Uppsala, Sweden). α-Calf serum and FCS were from HyClone (Thermo Fisher Scientific, Waltham MA); α-calf serum was heat inactivated at 56°C for 30 min before use. RPMI 1640 medium was from Life Technologies (Carlsbad, CA). EGTA, uric acid, MSU crystals, exo-ATPase inhibitor (ARL 67156), apyrase, ATP, ATP periodate oxidized sodium salt (oATP), ATP tetralithium salt (ATPγS), UTP, and Nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO). Uric acid was dissolved in the incubation medium at 1 mg/ml and diluted to desired concentrations. No crystal formation of uric acid was observed under an inverted microscope at the concentrations used in this study (≤0.1 mg/ml). The MSU crystals were suspended in the incubation buffer (e.g., RPMI 1640 medium) at 10 mg/ml and diluted serially. Suramin sodium salt was from EMD Chemicals (Gibbstown, NJ). KN-62 was from TOCRIS Bioscience (Ellisville, MO). Rabbit polyclonal anti-$P2Y_2$ Ab was from Thermo Fisher Scientific. Control rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell isolation

Eosinophils were isolated from the peripheral blood of 65 healthy volunteers, having no history of asthma or allergic diseases or having mild hay fever, by negative selection with anti-CD16 microbeads as previously described, with minor modifications (20). Briefly, granulocytes were incubated with an equal volume of anti-CD16–conjugated magnetic beads, FITC-conjugated anti-human CD14 mAb, and anti-FITC–conjugated magnetic beads on ice for 30 min. This protocol consistently yielded ≥99% eosinophil purity (mean, 99.4%). The responses of eosinophils from normal individuals and from patients with mild hay fever were not quantitatively different; therefore, the data were pooled. The Mayo Clinic Rochester Institutional Review Board approved the protocol to obtain blood from volunteers; all provided informed consent.

Eosinophil migration assay

Eosinophil migration through a membrane was examined using a 24-well Transwell insert system (Thermo Fisher Scientific) (21). These inserts with porous bottoms (pore = 3 μm) served as the upper chambers, and ordinary tissue-culture plate wells served as the lower chambers. Eosinophils were suspended in RPMI 1640 medium supplemented with 10% FCS at 1×10^6 cells/ml. One hundred microliters of the eosinophil suspension were added to the upper chamber, and 500 μl serial dilutions of uric acid solution or MSU crystal suspension were added to the lower chamber. Eotaxin (100 ng/ml) was used as a positive control. Alternatively, 100 μl eosinophil suspension with 0.1 mg/ml uric acid solution or MSU crystal suspension were added to the upper chamber, and 500 μl 0.1 mg/ml uric acid solution or MSU crystal suspension were added to the lower chamber in a checkerboard fashion. After 2 h of incubation at 37°C and 5% CO_2, the cells that migrated to the lower chambers were collected and counted by light microscopy. Results show the ratio (percentage) of migrated cells to the initial total number of cells.

Cytokine production

Purified eosinophils (1×10^6 cells/ml) were resuspended in RPMI 1640 with 10% FCS. Cells were incubated with medium alone or serial dilutions of MSU crystal suspensions or uric acid solution in 96-well tissue-culture plates for 24 h at 37°C and 5% CO_2 (22). After culture, supernatants were collected, and concentrations of IL-6 and IL-8 were measured by ELISA kits, according to the manufacturer's directions (R&D Systems). Sensitivities for IL-6 and IL-8 were 4 pg/ml. The supernatants were also analyzed by a Bio-Plex human 27 cytokine assay kit and the Bio-Plex suspension array system (Bio-Rad, Hercules, CA). All assays were conducted in duplicate. In some experiments, ATP (20–500 μM), ATPγS (20–500 μM), or UTP (100 μM) was used to stimulate eosinophils instead of MSU crystals, and the exo-ATPase inhibitor ARL 67156 (12.5–200 μM) was used to block the effects of endogenous ATPase.

To examine the role of endogenous ATP in the eosinophils' response to MSU crystals, pharmacological agents were added, including apyrase (5–20 U/ml), oATP (10–300 μM), or suramin (50–500 μM). To examine the role of the $P2Y_2$ nucleotide receptor in the eosinophils' cytokine responses to MSU crystals, eosinophils were preincubated with serial dilutions of rabbit polyclonal anti-$P2Y_2$ Ab or control rabbit IgG for 15 min before the addition of MSU crystal suspensions. To examine the involvement of the $P2X_7$ nucleotide receptor, eosinophils were preincubated with an antagonist for the $P2X_7$ receptor (KN-62; 50 nM) for 15 min before the addition of MSU crystal suspensions, ATP, or UTP.

Eosinophil degranulation assay

Eosinophil degranulation was quantitated by the eosinophil-derived neurotoxin (EDN) released into cell-free supernatants (23). Briefly, freshly isolated eosinophils were suspended in HBSS with 25 mM HEPES and 0.01% gelatin (3-h culture) or RPMI 1640 supplemented with 10 mM HEPES and 10% FCS (24-h culture) at 2.5×10^5 cells. Cells were added to the wells of 96-well tissue-culture plates and incubated with serial dilutions of MSU crystal suspensions (1–10 mg/ml), with or without 1 ng/ml IL-33 or 1 ng/ml IL-5, for 3 or 24 h at 37°C and 5% CO_2. After incubation, cell-free supernatants were collected and stored at −20°C before EDN was measured by ELISA. In some experiments, 1 mM EGTA was added to the culture to examine the role of extracellular calcium in eosinophil degranulation. To examine the role of P2Rs, eosinophils were preincubated with serial dilutions of oATP for 15 min before the addition of 3 mg/ml MSU crystal suspensions.

The EDN ELISA was performed as described earlier (24), using anti-human EDN mAbs (clones 167-6C5 and 167-2G4) made at Mayo Clinic Rochester. The lowest point of the standard curve was 0.09 ng/ml. All assays were conducted in duplicate.

Cell-viability assay

The viability of cultured eosinophils was assessed by double staining with FITC-conjugated Annexin V and propidium iodide (PI) and flow cytometry analysis. Briefly, eosinophils (5.0×10^5 cells) were suspended in HBSS buffer or RPMI 1640 medium, as described above, and incubated with medium alone or serial dilutions of MSU crystal suspensions or uric acid solutions in 5-ml polystyrene round-bottom tubes (BD Biosciences) for 3 or 24 h at 37°C and 5% CO_2. After washing, cells were suspended in binding buffer and stained with FITC-conjugated Annexin V and PI, according to the procedure recommended by the manufacturer (Annexin V-FITC Kit, Miltenyi Biotec). At least 10,000 cells were analyzed using flow cytometry (FACScan; BD Biosciences) and BD Biosciences Lysis II software. The percentages of apoptotic cells (Annexin V^+PI^-) and dead cells (Annexin V^+ PI^+) were determined. Alternatively, immediately after a 3 h-degranulation assay with MSU and oATP as described above, eosinophils were stained with PI and fluorescein diacetate (Sigma-Aldrich), and ≥200 cells were analyzed using a hemacytometer and an epifluorescent microscope.

Expression of $P2Y_2$ nucleotide receptor

Eosinophil surface expression of $P2Y_2$ nucleotide receptor was examined by flow cytometry. Purified eosinophils (1×10^6 cells) were resuspended in PBS with 3% BSA and 0.1% sodium azide (PAB) and incubated with rabbit anti-human $P2Y_2$ Ab or control rabbit IgG for 60 min on ice. After washing with PAB buffer, cells were incubated with FITC-conjugated $F(ab')_2$ of goat anti-rabbit IgG for 30 min on ice. Cells were washed twice with PAB, fixed with 1% paraformaldehyde for 20 min, and analyzed using flow cytometry (FACScan) and BD Biosciences Lysis II software.

ATP release

To examine ATP release by eosinophils, purified eosinophils were suspended at 1×10^6/ml in HBSS with 25 mM HEPES and 0.01% gelatin and incubated with medium alone or 0.1 mg/ml MSU crystal suspension in polypropylene tubes for up to 20 min at 37°C. After incubation, the reaction was stopped by centrifuging the tubes at 4°C and collecting the cell-free supernatants. To quantitate ATP release, the ATP concentrations in the supernatants were measured with an ATP Determination Kit (BioAssay Systems, Hayward, CA) and a luminometer.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA (Tukey–Kramer multiple comparisons test), or differences between two sample groups were analyzed with the paired Student *t* test. A p value <0.05 was considered statistically significant.

Results

Soluble uric acid and MSU crystals induce eosinophil chemotaxis

Sites of chronic inflammation or tissue fibrosis frequently contain eosinophils (4, 25, 26). In addition, eosinophils can accumulate even in the absence of acquired immune responses (26, 27) or before Ag sensitization (28). After dying cells release uric acid, MSU crystals have been identified as a danger signal (19). Therefore, we examined whether MSU crystals or soluble uric acid could induce eosinophil

migration. MSU crystal suspensions and uric acid solutions ≥ 0.03 mg/ml induced eosinophil migration ($p < 0.05$; $n = 5$), which seemed to increase with MSU or uric acid concentrations up to 0.1 mg/ml (Fig. 1*A*). The eosinophil migration induced by MSU crystals or uric acid was ~70% compared with an authentic eosinophil chemotactic factor (eotaxin; 100 ng/ml). Uric acid concentrations >0.1 mg/ml were not examined because the solute crystallized.

We used a checkerboard experiment to characterize eosinophil migration. Eosinophils with or without the MSU crystal suspension or uric acid solution (each at 0.1 mg/ml) were placed in the upper chamber, and the MSU crystal suspension or uric acid solution (0.1 mg/ml) was placed in the lower chamber. MSU crystals or uric acid in the lower chamber, but not in the upper chamber, induced eosinophil migration compared with medium alone ($p < 0.05$; $n = 5$) (Fig. 1*B*). MSU crystals or uric acid in the upper chamber abolished eosinophil migration to the lower chamber. Therefore, eosinophil migration likely requires a gradient of MSU crystals or uric acid.

MSU crystals, but not uric acid, induce robust cytokine production

To examine whether MSU crystals or uric acid induce eosinophil cytokine production, eosinophils were incubated with MSU crystals or uric acid, and the levels of IL-6 and IL-8 in the cell-free supernatants were measured by ELISA. MSU crystals induced robust production of IL-6 and IL-8 (Fig. 2). Cytokine production was observed with MSU crystals at 0.03 mg/ml (for IL-6) or 0.01 mg/ml (for IL-8), and it increased with concentrations of MSU crystals up to 0.1 mg/ml. In contrast, uric acid (≤0.1 mg/ml) did not induce IL-6 or IL-8 production.

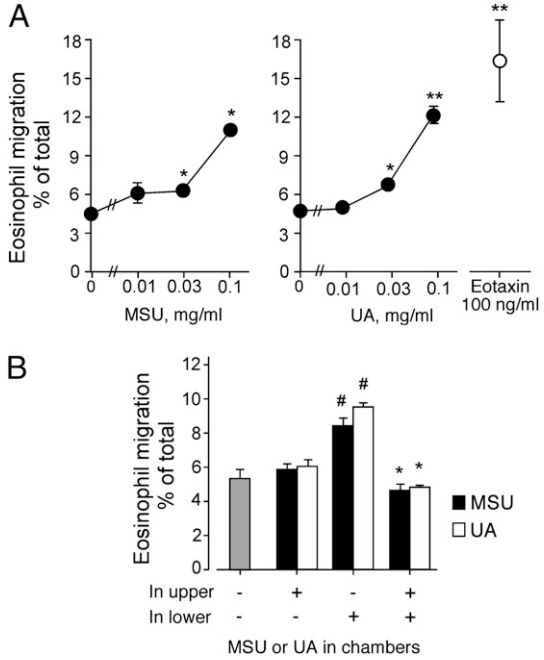

FIGURE 1. MSU crystals and soluble uric acid induce eosinophil chemotaxis. *A*, Human eosinophil suspensions were added to the upper chamber, and MSU crystal suspensions or uric acid solutions were added to the lower chamber. Eotaxin (100 ng/ml) in the lower chamber was used as a positive control. After 2 h at 37°C, the cells that migrated to the lower chambers were collected and counted with light microscopy. The data show the ratio (percentage) of migrated cells to the total number of input cells. Results show the mean ± SEM from five different eosinophil preparations. $*p < 0.05$; $**p < 0.01$, compared with medium alone in the lower chamber. *B*, Eosinophil suspensions with or without 0.1 mg/ml MSU crystals or uric acid were added to the upper chamber, and 0.1 mg/ml MSU crystals or uric acid were added to the lower chamber in a checkerboard fashion. After 2 h at 37°C, the cells in the lower chambers were collected and counted as in *A*; the data are summarized as in *A*. Results show the mean ± SEM from five different eosinophil preparations. $^{\#}p < 0.05$, compared with medium alone in upper and lower chambers; $*p < 0.05$, compared with MSU crystals or uric acid only in the lower chamber.

We used a Bio-Plex assay to examine a panel of cytokines and chemokines produced by eosinophils cultured with MSU crystal suspensions for 24 h. As shown in Table I, several cytokines were produced, including proinflammatory cytokines (IL-1β, IL-6, and TNF-α), a Th1 cytokine (IFN-γ), a Th17 cytokine (IL-17), a regulatory cytokine (IL-10), growth factors (G-CSF and GM-CSF), tissue-remodeling factors, vascular endothelial growth factor, TGF-β, and chemokines (CXCL8, CCL2, CCL3, and CCL4). Similar to the results for IL-6 and IL-8 (Fig. 2), the production of these cytokines and chemokines increased at MSU crystal concentrations of up to 0.1 mg/ml. In contrast, Th2 cytokines, such as IL-4, IL-5, and IL-13, and other cytokines and chemokines, including IL-2, IL-7, IL-9, IL-12p70, IL-15, CCL5, CCL10, CCL11, nerve growth factor, and platelet-derived growth factor-bb, were undetectable. Altogether, human eosinophils exposed in vitro to MSU crystals produced several cytokines, growth factors, and chemokines.

MSU crystals induced modest EDN release from eosinophils

We then examined whether eosinophils stimulated with MSU crystals release granule proteins. Eosinophils were stimulated with MSU crystal suspensions for 3 or 24 h, and EDN released into supernatants was measured by ELISA. MSU crystals induced modest EDN release, with ≥1 mg/ml by 3 h and ≥3 mg/ml by 24 h ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3*A*). The EDN release required ~10-fold more concentrated suspension of MSU crystals (Fig. 3) compared with the concentrations needed to induce chemotaxis or cytokine production (Figs. 1, 2). Furthermore, at the optimal MSU crystal concentrations (~3 mg/ml), the quantity of released EDN was ~3% of total EDN; this was considerably less than the percentage of total EDN typically induced by authentic secretagogues for human eosinophils (e.g., 1 μM platelet-activating factor induces 40% of total EDN) (29). Soluble uric acid at ≤0.1 mg/ml had no effect on EDN release (data not shown).

Because the EDN release induced by MSU crystals was modest, we investigated whether the MSU crystals mediated an unknown toxic or cytolytic effect. Thus, we examined the effects of IL-5 and IL-33, which also activate the effector functions of human eosinophils (24). Suboptimal concentrations of IL-5 alone (1 ng/ml) or IL-33 alone (1 ng/ml) induced modest EDN release (Fig. 3*B*). IL-5 and IL-33 enhanced EDN release induced by 1 or 10 mg/ml MSU crystals at 3 and 24 h,

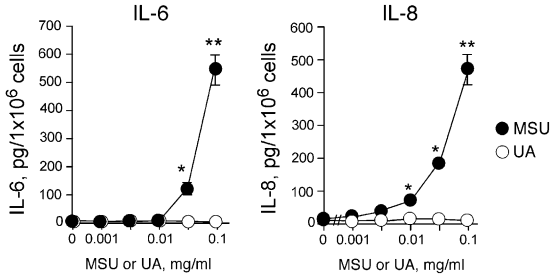

FIGURE 2. MSU crystals, but not soluble uric acid, induce IL-6 and IL-8 production by human eosinophils. Purified eosinophils were incubated with medium alone or serial dilutions of MSU crystal suspensions or uric acid (UA) solution for 24 h at 37°C. Concentrations of IL-6 and IL-8 in the cell-free supernatants were measured by ELISA. Results show the mean ± SEM from five different eosinophil preparations. $*p < 0.05$; $**p < 0.01$, compared with medium alone.

Table I. *Production of cytokines and chemokines by eosinophils stimulated with MSU crystals*

	MSU Crystals (mg/ml)		
	0	0.03	0.1
	Concentrations of Cytokines Detected (pg/ml)		
IL-1β	0 ± 0	27 ± 4*	57 ± 6**
IL-1Rα	1 ± 1	20 ± 4*	48 ± 4**
IL-6	0 ± 0	204 ± 30**	717 ± 101**
IL-8/CXCL8	29 ± 2	785 ± 129**	3840 ± 1220**
IL-10	0 ± 0	1 ± 0	9 ± 1**
IL-17	0 ± 0	1 ± 0	14 ± 4*
Fibroblast growth factor	4 ± 1	8 ± 2	23 ± 4*
G-CSF	0 ± 0	5 ± 1*	39 ± 6**
GM-CSF	1 ± 0	3 ± 1	16 ± 5*
IFN-γ	0 ± 0	5 ± 1*	29 ± 5**
MCP-1/CCL2	5 ± 1	20 ± 2*	47 ± 6**
MIP-1α/CCL3	0 ± 0	28 ± 5*	115 ± 18**
MIP-1β/CCL4	26 ± 2	585 ± 99*	2050 ± 516**
TNF-α	2 ± 1	35 ± 6*	118 ± 18**
Vascular endothelial growth factor	3 ± 0	6 ± 1*	17 ± 2*
TGF-β	10 ± 3	61 ± 13*	88 ± 7**

Eosinophils were incubated with medium alone or indicated concentrations of MSU crystal suspensions (0.03 or 0.1 mg/ml) for 24 h at 37°C. Concentrations of chemokines and cytokines in cell-free supernatants were determined by Bio-Plex. Results show the mean ± SEM from five different eosinophil preparations.

IL-2, IL-4, IL-5, IL-7, IL-9, IL-12 p70, IL-13, IL-15, RANTES/CCL5, IP-10/CXCL10, platelet-derived growth factor-bb, eotaxin/CCL11, and nerve growth factor were not detected in any sample.

*$p < 0.05$; **$p < 0.01$, compared with medium alone.

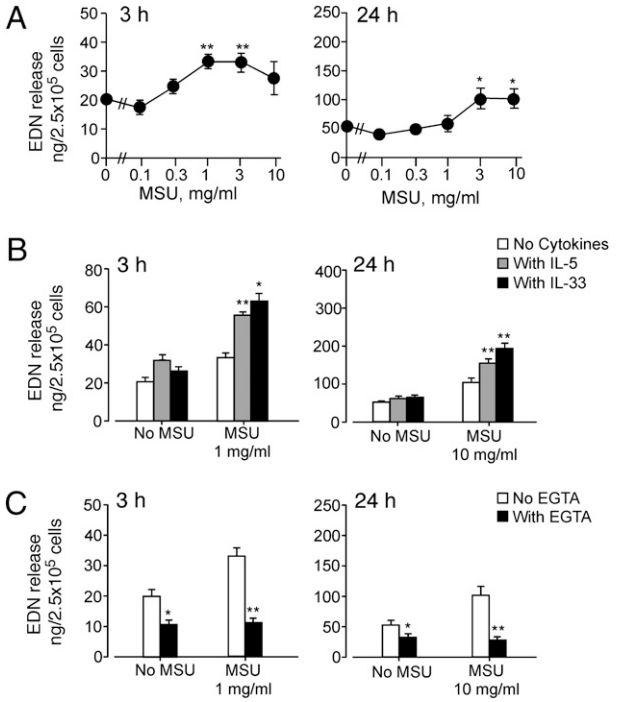

FIGURE 3. MSU crystals induce modest degranulation of eosinophils, which is regulated by cytokines and extracellular calcium. *A*, Eosinophils were incubated with MSU crystal suspensions for 3 or 24 h at 37°C. EDN released into supernatants was measured by ELISA. Results show the mean ± SEM from eight (for 3 h) or four (for 24 h) different eosinophil preparations. *$p < 0.05$; **$p < 0.01$, compared with medium alone. *B*, Eosinophils were incubated with or without 1 mg/ml (for 3 h) or 10 mg/ml (for 24 h) MSU crystal suspensions at 37°C with or without 1 ng/ml IL-33 or 1 ng/ml IL-5. EDN released into supernatants was measured by ELISA. Results show the mean ± SEM from eight (for 3 h) or four (for 24 h) different eosinophil preparations. *$p < 0.05$; **$p < 0.01$, compared with the samples with MSU but no cytokines. *C*, Eosinophils were incubated with or without 1 mg/ml (for 3 h) or 10 mg/ml (for 24 h) MSU crystal suspensions at 37°C with or without 1 mM EGTA. EDN released into supernatants was measured by ELISA. Results show the mean ± SEM from eight (for 3 h) or four (for 24 h) different eosinophil preparations. *$p < 0.05$; **$p < 0.05$, compared with the samples without EGTA.

respectively ($p < 0.05$ or $p < 0.01$). Furthermore, increased intracellular calcium concentration is a key triggering step in the coupling of stimulus to eosinophil degranulation (30). When we used 1 mM EGTA to chelate extracellular calcium, eosinophils did not degranulate in response to MSU crystals (Fig. 3*C*). These findings suggest that eosinophil degranulation induced by MSU crystals is controlled under physiological conditions (e.g., cytokines or extracellular calcium).

Exposure to MSU crystals modulates eosinophil viability

Eosinophil cytokine production declined when cells were incubated with >1 mg/ml MSU crystal suspensions (Fig. 2). Therefore, we examined the effects of different concentrations of MSU crystals on eosinophil viability. Incubation of eosinophils for 3 h with medium alone or MSU crystals at 0.01 or 0.1 mg/ml did not affect eosinophil viability (Fig. 4*A*); ≤2.9% and 0.2% of the cells were apoptotic and dead, respectively. In contrast, MSU crystals at 3 or 10 mg/ml increased the proportions of apoptotic and dead cells ($p < 0.01$; $n = 5$). When eosinophils were incubated with medium alone for 24 h, the proportion of apoptotic cells increased to ~13% of total cells, consistent with increased apoptosis of eosinophils when they are cultured without any growth factors for a prolonged period (31). This increase in eosinophil apoptosis at 24 h was partially inhibited by 0.1 mg/ml MSU crystals ($p < 0.01$; $n = 5$). In contrast, MSU crystals at ≥1 mg/ml increased the proportion of dead cells at 24 h. Soluble uric acid at concentrations ≤0.1 mg/ml showed no effects on eosinophil viability (Fig. 4*B*). Thus, MSU crystals at low concentrations (e.g., 0.1 mg/ml) likely inhibit eosinophil apoptosis and promote cytokine production, but at higher concentrations they induce cell death.

Endogenous ATP is involved in the eosinophils' responses to MSU crystals

What could be the molecular mechanisms involved in the eosinophils' responses to MSU crystals? No cellular receptors for MSU crystals have been identified (32). Interestingly, endogenous ATP is critically involved in cytokine production by monocytes stimulated with TLR ligands (33) and in the directed migration of neutrophils induced by FMLP (34). Therefore, we hypothesized that autocrine ATP is involved in the eosinophils' responses to MSU crystals.

To test the hypothesis, we first measured the concentrations of ATP in the culture supernatants of eosinophils. ATP was detectable within 1 min after placing the eosinophils in a test tube with medium alone (Fig. 5*A*); ATP levels returned to baseline levels within 5 min, likely as a result of the effects of ecto-ATPase activity. Importantly, when MSU crystals were added to eosinophils, the peak extracellular ATP level was ~3-fold greater than for eosinophils with medium alone, and these detectable levels of ATP were more persistent. Thus, eosinophils exposed to MSU crystals quickly release ATP.

We next examined whether this released ATP is involved in cytokine production by eosinophils stimulated with MSU crystals. To this end, we neutralized the increases in ATP by exogenously administering an ATP-hydrolyzing enzyme apyrase (33, 35). Apyrase partially (~50%), but significantly, inhibited IL-6 production by eosinophils stimulated with MSU crystals ($p < 0.05$) (Fig. 5*B*). We next examined the effects of broad-range inhibitors of P2Rs, as receptors for ATP. oATP is a derivative of ATP, and it covalently modifies nucleotide-binding proteins

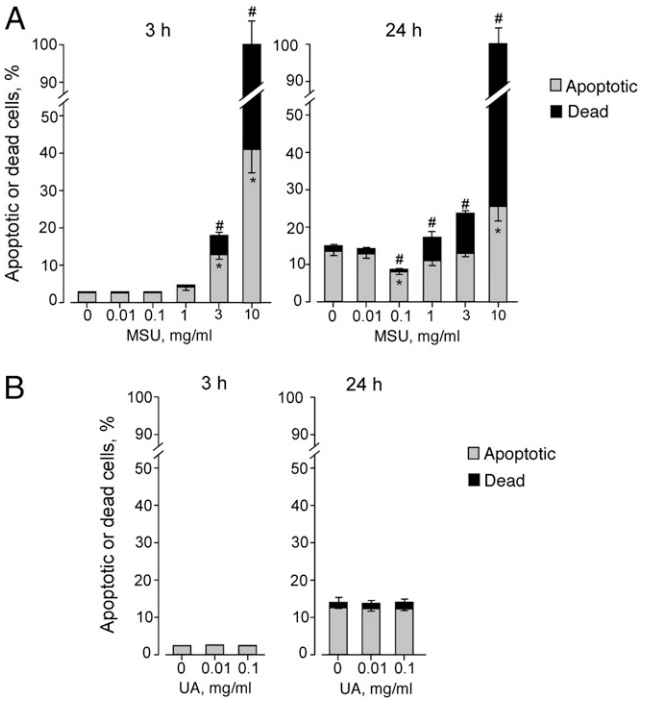

FIGURE 4. MSU crystals modulate eosinophil viability. *A*, Eosinophils were incubated with medium alone or serial dilutions of MSU crystal suspensions for 3 or 24 h at 37˚C. *B*, Eosinophils were incubated with medium alone or serial dilutions of soluble uric acid (UA) for 3 or 24 h at 37˚C. Cell apoptosis and death were examined by double staining with FITC-conjugated Annexin V and PI and flow cytometry analysis. The percentages of apoptotic cells (Annexin V^+PI^-) and dead cells (Annexin V^+ PI^+) were determined. The proportion of Annexin V^-PI^+ cells was <0.5%. Results show the mean ± SEM from five (*A*) or three (*B*) different eosinophil preparations. Downward error bars and upward error bars indicate SEM for apoptotic cells and dead cells, respectively. $*p < 0.01$, compared with percentage of apoptotic cells in medium alone; $^{\#}p < 0.01$, compared with percentage of dead cells in medium alone.

(33, 35). oATP potently inhibited IL-6 production by eosinophils stimulated with MSU crystals with an IC_{50} ~20 μM (Fig. 5*B*). Suramin is a broad-spectrum antagonist for P2Rs (36, 37), and it strongly inhibited IL-6 production by eosinophils (IC_{50} 50 μM) and abolished the response at 500 μM (Fig. 5*B*). Thus, autocrine ATP interacting with P2Rs is likely involved in the MSU crystal-induced production of IL-6 by eosinophils.

We also examined whether the increased extracellular ATP levels around the eosinophils in media only (without MSU crystals) are sufficient to induce cytokine production. Addition of exogenous ATP or ATPγS alone to eosinophils induced robust IL-6 production (Fig. 6*A*). Furthermore, inhibition of the eosinophils' ATPase activity by an ecto-ATPase inhibitor (ARL 67156) enhanced IL-6 production (without MSU crystals) ($p < 0.05$; $n = 5$) (Fig. 6*B*). Thus, the ability of eosinophils to produce IL-6 seems to be tightly regulated by the extracellular levels of ATP and the activity of a catalytic enzyme(s).

$P2Y_2R$ is likely involved in the eosinophils' cytokine responses to ATP and MSU crystals

Because oATP and suramin may block receptors in addition to P2Rs (e.g., the sphingosine-1-phosphate receptor-3) (36), we used an Ab and pharmacological agents to dissect the role for specific P2Rs in the eosinophils' responses to ATP and MSU crystals. Several nucleotide receptors (P2X and P2Y subfamilies) have been detected in human eosinophils by mRNA levels (38). $P2Y_2R$ has been implicated in neutrophil chemotaxis mediated by endogenous ATP (34). Therefore, we suspected a role for $P2Y_2R$ in the eosinophils' response to ATP. Flow cytometry consistently detected the surface expression of $P2Y_2$ protein in freshly isolated human eosinophils from six donors (Fig. 7*A*).

To examine the role of $P2Y_2R$ in the eosinophils' response to ATP, we incubated eosinophils with anti-$P2Y_2$ Ab or control Ab and stimulated the cells with 100 μM ATP. Anti-$P2Y_2$ Ab nearly abolished ATP-induced IL-6 production, but the control Ab did not (Fig. 7*B*), suggesting that $P2Y_2$ is involved in the eosinophils' IL-6 production in response to exogenous ATP. Furthermore, UTP, an agonist for $P2Y_2R$ and $P2Y_4R$ (39), potently induced IL-6 production, which was also abolished by anti-$P2Y_2$ Ab (Fig. 7*B*). Therefore, perturbation of eosinophil $P2Y_2$ by exogenous ATP or UTP is likely sufficient to induce IL-6 production. In contrast, neither ATP nor UTP showed significant effects on IL-8 production (Fig. 7*B*).

To examine the role of $P2Y_2R$ in the eosinophils' IL-6 and IL-8 production in response to MSU crystals, we incubated eosinophils with

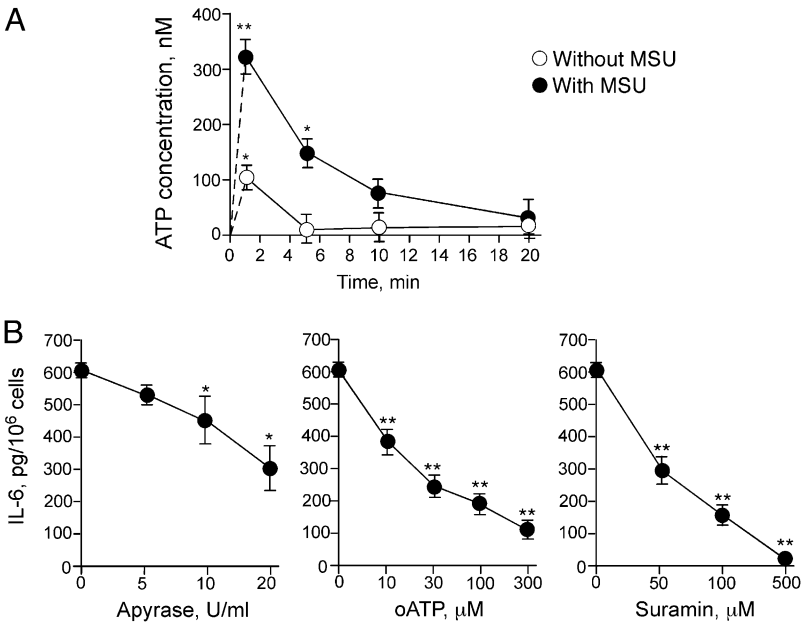

FIGURE 5. Autocrine release of ATP by MSU-stimulated eosinophils is involved in IL-6 production. *A*, Eosinophils were incubated with medium alone or 0.1 mg/ml MSU crystal suspension in polypropylene tubes for up to 20 min at 37˚C. The reaction was stopped by centrifugation at 4˚C. The concentrations of ATP in the supernatants were measured using an ATP Determination Kit. Results show the mean ± SEM from three different eosinophil preparations. $*p < 0.05$; $**p < 0.05$, compared with samples without MSU. *B*, Eosinophils were incubated with 0.1 mg/ml MSU crystal suspensions with or without serial dilutions of apyrase (5–20 U/ml), oATP (10–300 μM), or suramin (50–500 μM) for 24 h at 37˚C. Concentrations of IL-6 in supernatants were measured by ELISA. Results show the mean ± SEM from three to six different eosinophil preparations. $*p < 0.05$; $**p < 0.05$, compared with samples without inhibitors.

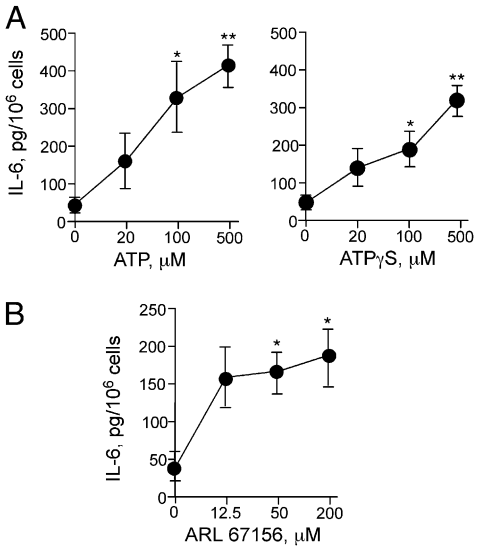

FIGURE 6. Exogenous ATP and inhibition of exo-ATPase activity trigger IL-6 production by eosinophils. *A*, Eosinophils were incubated with ATP or ATPγS for 24 h at 37°C. Concentrations of IL-6 in cell-free supernatants were measured by ELISA. Results show the mean ± SEM from five different eosinophil preparations. $*p < 0.05$; $**p < 0.01$, compared with medium alone. *B*, Eosinophils were incubated with an exo-ATPase inhibitor (ARL 67156) for 24 h at 37°C. Concentrations of IL-6 in cell-free supernatants were measured by ELISA. Results show the mean ± SEM from five different eosinophil preparations. $*p < 0.05$, compared with medium alone.

MSU crystals in the presence of anti-$P2Y_2$ Ab or control Ab. Anti-$P2Y_2$ Ab inhibited the eosinophils' production of IL-6 and IL-8; control Ab showed minimal effects (Fig. 7*C*). An antagonist for $P2X_7R$ (KN-62; 50 nM) showed no effects on IL-6 or IL-8 production induced by MSU crystals (data not shown). Altogether, by recognizing autocrine ATP, $P2Y_2$ likely plays a pivotal role in the eosinophils' cytokine response to MSU crystals.

ATP-mediated autocrine feedback loops are likely involved in MSU-induced eosinophil degranulation

Because eosinophil viability decreased when cells were incubated with higher concentrations of MSU crystals (e.g., ≥1 mg/ml) (Fig. 4), we asked whether the EDN released at these concentrations of MSU (Fig. 3) was a result of passive cell death or ATP-mediated cell activation. To address this, we examined the effects of a broad-range inhibitor of P2Rs (i.e., oATP) on EDN release and cell viability. Eosinophils incubated with 3 mg/ml MSU crystals showed significant EDN release and increased cell death compared with those incubated without MSU ($p < 0.05$) (Fig. 8). This MSU-induced EDN release was significantly inhibited by oATP in a concentration-dependent manner, suggesting that P2Rs are involved in EDN release. Importantly, cell death was also inhibited by treating the cells with oATP, suggesting that loss of cell viability is also mediated by P2Rs. These findings suggest that, at high concentrations, MSU crystals promote P2R-dependent release of the eosinophil granule protein, EDN, as well as cell death.

Discussion

When we exposed human eosinophils to MSU crystals, these cells produced abundant quantities of IL-6 and several other cytokines and chemokines, including Th1 cytokines, proinflammatory cytokines, and chemokines, but no Th2 cytokines. Autocrine production of ATP likely plays a pivotal role in this process by providing a positive feedback, as demonstrated by the inhibitory effects of exogenous ATPase, broad-spectrum P2 antagonists, and anti-$P2Y_2$ Ab. Addition of exogenous ATP or blockade of endogenous ATPase activity also induced IL-6 production by eosinophils.

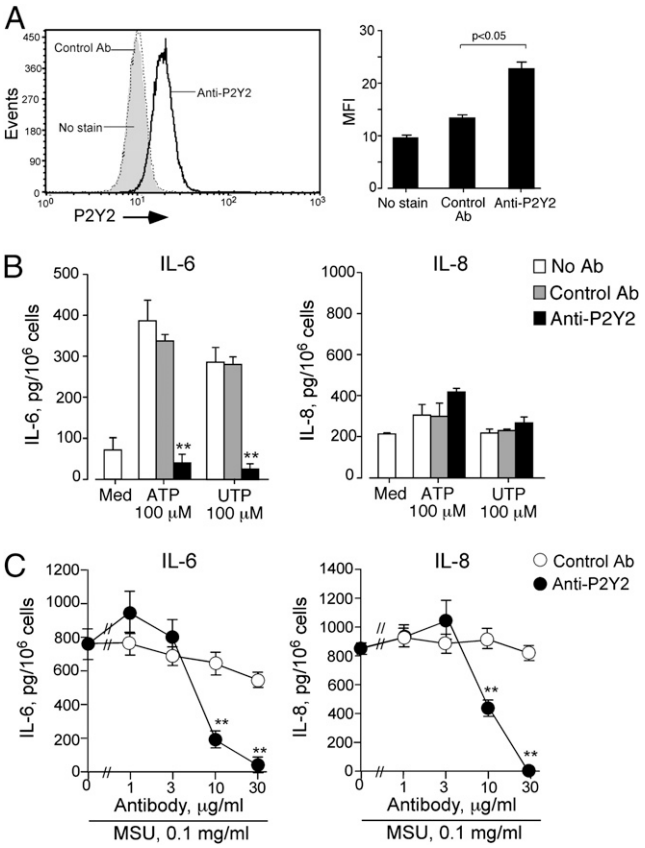

FIGURE 7. Human eosinophils express $P2Y_2R$, and anti-$P2Y_2$ Ab blocks the eosinophils' responses to exogenous ATP, UTP, and MSU crystals. *A*, Eosinophils were incubated with rabbit anti-human $P2Y_2$ Ab or control Ab. Cells were then incubated with FITC-conjugated goat anti-rabbit IgG, fixed with 1% paraformaldehyde, and analyzed by flow cytometry. *Left panel* shows a representative graph. *Right panel* shows a summary with eosinophils from six different donors. Results show the mean ± SEM of mean fluorescence intensity (MFI). *B*, Eosinophils were preincubated with or without rabbit polyclonal anti-$P2Y_2$ Ab or control Ab (20 μg/ml) for 15 min and cultured with medium alone, 100 μM ATP, or 100 μM UTP for 24 h at 37°C. Concentrations of IL-6 and IL-8 in the supernatants were measured by ELISA. Results show the mean ± SEM from five different eosinophil preparations. *C*, Eosinophils were preincubated with or without anti-$P2Y_2$ Ab or control Ab (1–30 μg/ml) for 15 min and cultured with 0.1 mg/ml MSU crystal suspension for 24 h at 37°C. Concentrations of IL-6 and IL-8 in the supernatants were measured by ELISA. Results show the mean ± SEM from six different eosinophil preparations. $**p < 0.01$, compared with control Ab.

MSU crystals have been identified as a danger signal formed after the release of uric acid from dying cells or damaged tissues (19, 40). The physiological significance of these eosinophil responses to MSU crystals or damaged tissues remains open for speculation. Eosinophils may participate in immunological surveys to recognize tissue damage or injury. For example, in mice injected with B16 melanoma cells, infiltration by eosinophils was observed in the necrotic regions of tumors, even without acquired immune responses (26, 27). In naive mice injected i.p. with aluminum hydroxide, splenic eosinophils were recruited, and these eosinophils primed the early Ag-specific IgM Ab response (28). More recently, eosinophils were shown to recognize a prototypic DAMP molecule, HMGB-1, which is released by necrotic cells (41). Eosinophils release various cytokines (5, 42), and eosinophil-derived factor(s) may be involved in the recruitment of Th2-type $CD4^+$ T cells in mouse models of allergic

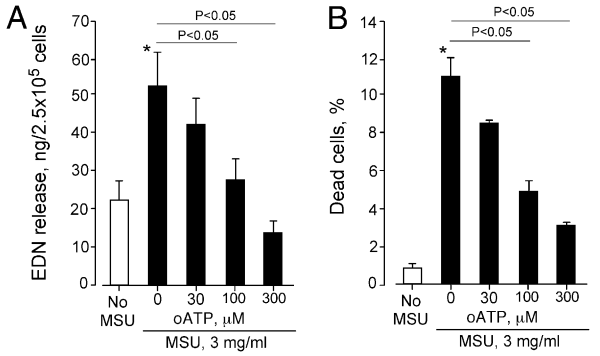

FIGURE 8. Blockade of P2Rs with oATP inhibits EDN release and decreases cell death induced by MSU crystals. Eosinophils were preincubated with serial dilutions of oATP for 15 min and incubated with or without 3 mg/ml MSU crystal suspensions for 3 h at 37°C. *A*, EDN released into supernatants was measured by ELISA. *B*, Eosinophil viability was examined by staining cells with PI and fluorescein diacetate and by analyzing ≥200 cells with an epifluorescent microscope. The percentages of dead cells (PI^+Fluorescein $diacetate^-$) were determined. Results show the mean ± SEM from five different eosinophil preparations. $*p < 0.05$, compared with no MSU.

airway inflammation (8, 9). Furthermore, eosinophils have an Ag-presenting capability (43). Therefore, reacting to a danger signal, such as MSU crystals and HMGB-1, may be a part of the eosinophils' innate immune responses, leading to the regulation of adaptive immunity in a Th2-type environment. Furthermore, the chronic and waxing-and-waning nature of eosinophilic airway diseases, such as asthma and chronic rhinosinusitis, may be explained by a self-perpetuating mechanism involving tissue injury, release of damage-associated molecules, and eosinophil infiltration and inflammatory mediator release.

Extracellular ATP has gained attention as a mediator of intercellular communication and an autocrine mediator (44, 45). An in vivo mouse model of asthma implicated extracellular ATP in the induction and maintenance of Th2-type airway inflammation (46). The intracellular concentration of ATP ranges between 5 and 10 mM, and cell death or even cellular stress releases large amounts of ATP into the pericellular space (47, 48). Although information is limited regarding triggers for ATP release by inflammatory cells (33), we observed rapid ATP release (within 1 min) from eosinophils exposed to MSU crystals. A nonlytic mechanism likely mediates this fast response, because when eosinophils are exposed to MSU crystals and cultured for 24 h, they synthesize new proteins (i.e., IL-6 and IL-8) (Fig. 2). Furthermore, eosinophil viability was not compromised by the concentration of MSU crystals (i.e., 0.1 mg/ml) that induced robust ATP release (Figs. 4, 5). A recent study suggested that MSU crystals induce the aggregation of cellular membranes, particularly the cholesterol components, without involving specific cellular receptors (32). Membrane deformation caused by mechanical stress induces the release of cellular ATP from mammalian cells (49, 50). Eosinophils exposed to MSU crystals may release ATP as a result of cellular stress caused by alterations in cell-membrane structure. The extracellular concentration of ATP is tightly regulated by ubiquitous ecto-ATPase activity (47). Because the addition of an ecto-ATPase inhibitor (ARL 67156) alone was sufficient to induce IL-6 production (Fig. 6) without any additional stimuli, eosinophils are themselves tightly regulated. Thus, a fine balance between stress-induced ATP release and ATP catalysis likely plays a pivotal role in regulating the eosinophils' cytokine production. It remains unknown whether the stress status of eosinophils or the reduced activity of ecto-ATPase activity or both may explain the increased effector functions of eosinophils in diseased organs (51).

Because suramin and oATP broadly block P2Rs, the exact P2Rs involved in mediating the effects of autocrine ATP in eosinophils remain to be elucidated. Expression of a P2R $P2Y_2$ was clearly observed on the cell surface of freshly isolated eosinophils (Fig. 7*A*). Exogenous ATP and UTP induced robust IL-6 production (Fig. 7*B*), which was abolished by anti-$P2Y_2$ Ab. In contrast, previous findings showed that exogenous ATP, but not UTP, induced IL-8 production by eosinophils, which was partially inhibited by a $P2X_7$ antagonist KN-62 (52). Therefore, the agonists and intracellular signaling mechanisms required for IL-6 and IL-8 production by eosinophils are probably different. Interestingly, IL-6 and IL-8 production induced by MSU crystals were inhibited by anti-$P2Y_2$ Ab. Thus, activation of $P2Y_2$ by endogenous ATP released by MSU-exposed eosinophils is likely sufficient to provoke robust IL-6 production. In contrast, endogenous ATP may be necessary but insufficient to induce robust IL-8 production. Altogether, our observations suggest a pivotal role for $P2Y_2$ in the eosinophils' responses to endogenous and exogenous ATP and subsequent production of certain cytokines. These results need to be verified when more selective P2R agonist/antagonist and/or gene-deficient animals become widely available. Human eosinophils express mRNA for $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{11}$, and $P2Y_{14}$ (38, 53–55). For technical reasons, the small interfering RNA knockdown of specific mRNA has been unsuccessful in human eosinophils (data not shown).

Eosinophils release EDN in response to relatively high concentrations (i.e., ≥1 mg/ml) of MSU crystals (Fig. 3). The magnitude of EDN release in response to MSU crystals was small and ~10-fold less than that induced by authentic agonists (e.g., platelet-activating factor) for human eosinophils. Nonetheless, MSU-induced EDN release is likely mediated by autocrine ATP, similarly to cytokine production, because blockade of P2Rs by oATP significantly inhibited EDN release (Fig. 8). Increased cell viability when eosinophils are pretreated with oATP also suggests that eosinophil cell death induced by MSU crystals is likely the result of ATP-mediated cell activation and degranulation, but is probably not due to direct cytotoxicity by MSU crystals. Eosinophil degranulation activated by physiologic or immunological stimuli, such as immobilized secretory IgA or ligation of FcγRII, induces cell death (56, 57). Thus, eosinophil exposure to MSU crystals likely induces migration, robust cytokine production, and modest degranulation. Indeed, dissociation between cytokine production and EDN release was observed when eosinophils were incubated with soluble IgA immune complex or stimulated with ligation of FcγRII by soluble Ab (56, 57).

Overall, preformed MSU crystals were highly stimulatory for cytokine production by eosinophils (Fig. 2), whereas soluble uric acid was not. Thus, ATP release and cytokine production probably requires physical interaction between particulate MSU crystals and eosinophils. Interestingly, soluble uric acid induced eosinophil chemotaxis in a gradient-dependent manner (Fig. 1). Uric acid is relatively insoluble in sodium-rich biological fluids (up to 70 μg/ml); uric acid remains soluble in certain buffers up to 1600 μg/ml (19, 58, 59). Because the cytosol contains high concentrations of uric acid (i.e., 4000 μg/ml) (19), the local environmental around dying cells should become supersaturated with uric acid when cells lyse, favoring the formation of MSU crystals. Our data suggest that a chemical phase transition of uric acid could be key in triggering distinct cellular functions (e.g., chemotaxis versus cytokine production) of human eosinophils. Perhaps, soluble uric acid in tissues relatively remote from dying cells is chemotactic, but MSU crystals in close proximity to the dying cells cause cytokine production.

In conclusion, human eosinophils respond to an endogenous danger signal, MSU crystals, resulting in robust production of a wide variety of cytokines and chemokines. Finding the precise

P2XRs and P2YRs involved in the eosinophils' recognition of autocrine ATP in mediating these responses will be challenging, but it could lead to new therapeutic approaches that suppress eosinophilic inflammation and disease pathology.

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Disclosures

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