Impaired P2X1 Receptor–Mediated Adhesion in Eosinophils from Asthmatic Patients

Adam Wright, Martyn Mahaut-Smith, Fiona Symon, Nicolas Sylvius, Shaun Ran, Mona Bafadhel, Michelle Muessel, Peter Bradding, Andrew Wardlaw and Catherine Vial

J Immunol 2016; 196:4877-4884; Prepublished online 9 May 2016;
doi: 10.4049/jimmunol.1501585
http://www.jimmunol.org/content/196/12/4877

Supplementary Material  http://www.jimmunol.org/content/suppl/2016/05/07/jimmunol.150158
5.DCSupplemental

References  This article cites 55 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/196/12/4877.full#ref-list-1

Why The JI?  Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2016 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Impaired P2X1 Receptor–Mediated Adhesion in Eosinophils from Asthmatic Patients

Adam Wright,* Martyn Mahaut-Smith,† Fiona Symon,**† Nicolas Sylvius,*
Shaun Ran,*† Mona Bafadhel,*§ Michelle Muesse,*§ Peter Bradding,*† Andrew Wardlaw,*†
and Catherine Vial*†**

Eosinophils play an important role in the pathogenesis of asthma and can be activated by extracellular nucleotides released following cell damage or inflammation. For example, increased ATP concentrations were reported in bronchoalveolar lavage fluids of asthmatic patients. Although eosinophils are known to express several subtypes of P2 receptors for extracellular nucleotides, their function and contribution to asthma remain unclear. In this article, we show that transcripts for P2X1, P2X4, and P2X5 receptors were expressed in healthy and asthmatic eosinophils. The P2X2 receptor agonist α,β-methylene ATP (α,β-meATP; 10 μM) evoked rapidly activating and desensitizing inward currents (peak 18 ± 3 pA/pF at −60 mV) in healthy eosinophils, typical of P2X1 homomeric receptors, which were abolished by the selective P2X1 antagonist NF449 (1 μM) (3 ± 2 pA/pF). α,β-meATP–evoked currents were smaller in eosinophils from asthmatic patients (8 ± 2 versus 27 ± 5 pA/pF for healthy) but were enhanced following treatment with a high concentration of the nucleotidase apyrase (17 ± 5 pA/pF for 10 IU/ml and 11 ± 3 pA/pF for 0.32 IU/ml), indicating that the channels are partially desensitized by extracellular nucleotides. α,β-meATP (10 μM) increased the expression of CD11b activated form in eosinophils from healthy, but not asthmatic, donors (143 ± 21% and 108 ± 11% of control response, respectively). Furthermore, α,β-meATP increased healthy (18 ± 2% compared with control 10 ± 1%) but not asthmatic (13 ± 1% versus 10 ± 0% for control) eosinophil adhesion. Healthy human eosinophils express functional P2X1 receptors whose activation leads to eosinophil α5β1 integrin–dependent adhesion. P2X1 responses are constitutively reduced in asthmatic compared with healthy eosinophils, probably as the result of an increase in extracellular nucleotide concentration. The Journal of Immunology, 2016, 196: 4877–4884.

Asthma is a common chronic inflammatory airway disease that causes considerable morbidity. It is defined by variable airflow obstruction associated with airway hyperresponsiveness and an inflammatory process that is often, although not invariably, eosinophilic (1). The close association between eosinophils and asthma has been noted for many years, and it is emerging that eosinophils play a causal role in severe exacerbations of the disease, although their importance in the development of airway hyperresponsiveness and variable airflow obstruction is less certain (2). Eosinophils may also contribute to airway tissue repair and remodeling (3). The physiological regulation of eosinophil functions (e.g., priming, activation, and migration) involves a broad range of cell surface receptors, including chemokine, cytokine and inhibitory receptors, innate and Fc receptors and adhesion molecules (3), as well as P2 receptors for nucleotides (4).

Extracellular nucleotides (e.g., ATP and ADP) act via cell surface P2 receptors, a family composed of P2X ligand–gated ion channels (P2X1–7) (5) and metabotropic P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14) (6). Functional P2X receptors are trimers that can be homomeric or heteromeric, thereby increasing the number of potential P2X receptor subtypes that can contribute to cell activity (5, 7, 8). An increase in extracellular nucleotide concentration occurs following cell degranulation, tissue injury, apoptosis, osmotic shock, mechanical stress, hypoxia, and inflammation (9–11). In human airways, nucleotides can be derived from damaged airway epithelium, airway smooth muscle, the accumulation and activation of diverse immune cells (e.g., mast cells, T lymphocytes, eosinophils, and neutrophils) (12), and activated platelets (13). Increased ATP concentrations in bronchoalveolar lavage fluids were reported from chronic smokers (14) and patients with asthma (15), chronic obstructive pulmonary disease (14), cystic fibrosis (16), and idiopathic pulmonary fibrosis (17).

Transcripts for several P2 receptors were reported in human eosinophils, including P2X1, P2X4, P2X5, and P2X7 receptors; however, the presence of P2X7 mRNA in resting eosinophils remains controversial (18, 19). Extracellular nucleotides elevate...
Table I. Clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Asthmatic Patients (n = 62)</th>
<th>Healthy Volunteers (n = 57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (a [%])</td>
<td>29 (53)</td>
<td>41 (72)</td>
</tr>
<tr>
<td>Age (y; mean [range])</td>
<td>55 (23–79)</td>
<td>47 (18–68)</td>
</tr>
<tr>
<td>Age at diagnosis (y; mean [range])</td>
<td>24 (0–65)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Atopy (a [%])</td>
<td>52 (84)</td>
<td>n.a.</td>
</tr>
<tr>
<td>FEV₁, FRC (%)</td>
<td>2.3 (0.1)</td>
<td>n.d.</td>
</tr>
<tr>
<td>FEV₁/FVC (%)</td>
<td>83 (3.6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Inhaled corticosteroid dose (µg)b</td>
<td>51 (4.6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Percentage with GINA 2/3</td>
<td>44</td>
<td>n.d.</td>
</tr>
<tr>
<td>Percentage with GINA 4/5</td>
<td>56</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Asthmatic patients and healthy control volunteers were ≥18 y.

Data are mean (SEM), unless stated otherwise.

béactin, bP2X1 –actin,

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; GINA 2, mild persistent; GINA 3, moderate persistent; GINA 4 and 5, severe persistent; n.a., not applicable; n.d., not determined.

eosinophil cytosolic calcium (18–21) and aggravate inflammatory states via the generation and release of cytotoxic eosinophil cationic protein, IL-5, and reactive oxygen species (ROS), in addition to upregulation of the integrin CD11b, enhanced chemotaxis, and eosinophil actin reorganization (18, 21–23). Allergen stimulation can upregulate P2Y2 receptor expression in eosinophils from patients with allergic asthma (24). Eosinophils isolated from P2Y2 receptor–deficient mice no longer migrate toward ATP, suggesting the importance of this receptor for eosinophil chemotaxis. P2X receptor(s) also were proposed to modulate eosinophil function; however, because of the indirect methods of characterization, the subtype(s) of receptor(s) involved are unclear (18, 19, 21–23).

The aims of this study were to characterize the P2X receptor(s) present on eosinophils from healthy and asthmatic donors and determine their contribution to eosinophil function.

Materials and Methods

Reagents

All reagents were from Sigma-Aldrich (Poole, U.K.), unless otherwise stated. 4,4′,4′-4′′-4′′′-Carbonylbis((imino-5,1,3-benzenetriyl-bis(carbonylimino)))tetrakis-1,3-benzenedisulfonic acid (NF449) and platelet-activating factor (PAF) were from Tocris Biosciences (Bristol, U.K.). Fura-2, AM, RPMI 1640 medium + GlutaMAX I, and FCS were obtained from Life Technologies (Paisley, U.K.), and IL-5 was purchased from R&D Systems Europe (Abingdon, U.K.).

Eosinophil isolation

Asthmatic patients, with a physician diagnosis of persistent asthma, as per Global Initiative for Asthma (GINA) guidelines (25), were recruited from the specialist airways clinics at Glenfield Hospital (Leicester, U.K.). Asthmatic patients were stable with no recent exacerbation. Table I summarizes the clinical characteristics of the blood volunteers recruited for the present study. This work was approved by the Leicestershire, Rutland, and Northamptonshire Health Ethics committee. Human eosinophils were isolated, as previously described (26), and resuspended in buffer W (RPMI 1640 medium + GlutaMAX I + 2% FCS). Purity was 99.4 ± 0.0% (99 donors).

RT-PCR and real-time PCR

Total RNA from eosinophils was isolated using the QIAshredder and RNase kit with on-column DNase I digestion (QIAGEN, Crawley, U.K.). Total RNA (5.6 ng) was reverse transcribed using the Sensiscript Reverse Transcription kit (QIAGEN) in 20 µl. For negative control, the Sensiscript enzyme was replaced with H₂O. PCR reactions using BIOTAQ DNA Polymerase (1.25 U/reaction; Bioline, London, U.K.) were performed (27) with 2 µl cDNA or negative control. Real-time PCR (qPCR) was conducted using 2.5 µl iQ SYBR Green qPCR Kit (Bio-Rad, Hemel Hempstead, U.K.). 1 µl forward/reverse primer mix (0.75 µM each), and 1.5 µl cDNA in a LightCycler 480 real-time PCR System (Roche Diagnostics, Burgess Hill, U.K.). qPCR expression data were normalized to the geometric mean of b-actin, bPPIB, and RPL13A1 mRNAs as a normalization factor using the 2⁻ΔCt method. The primer list is shown in Supplemental Table I.

Patch-clamp recording

Resuspended eosinophils were plated onto glass coverslips and incubated at 37°C for ≥30 min with apyrase grade VII to prevent receptor desensitization by endogenous ATP release. The apyrase concentration was 0.32 IU/ml (concentration previously shown to prevent complete desensitization of platelet P2X1 responses) (28) or 10 IU/ml. Conventional whole-cell patch-clamp recordings were made at a holding potential of ~60 mV at 21°C. The agonist was applied rapidly via a U-tube. To examine the effect of NF449 on agonist-evoked P2X1 receptor currents, cells were incubated for 5 min with the antagonist before concomitant application of the agonist.

Western blot

Eosinophil proteins were analyzed by Western blot (30). Then the membrane was stripped of bound Abs and reprobed with an anti-actin Ab (1:1000; Abcam, Cambridge, U.K.), followed by an HRP-conjugated goat anti-mouse IgG (1:5000 dilution; Jackson ImmunoResearch Europe, Suffolk, U.K.) using the same procedure as above. A total of 9 µg eosinophil protein sample was analyzed per donor for P2X1 protein expression. Human embryonic kidney cells (HEK293) stably transfected with P2X1 receptor expressed a very high level of P2X1 protein. Therefore, to avoid the obliteration of P2X1 protein signal in the neighboring samples on the
autoradiography film, we used 0.2 μg HEK cell protein/sample. Once P2X1 protein was analyzed, the hybridization membrane was stripped and reprobed for actin. Although 9 μg eosinophil protein sample/donor was adequate to detect a signal for actin protein, 0.2 μg protein for HEK cells was not sufficient to observe the actin signal on the autoradiography film.

Flow cytometric detection of eosinophil P2X1 total protein expression

Whole blood (100 μl) was stained with PE–Siglec-8 (1.5 μg/ml, 347104; BioLegend UK, London, U.K.) and PE–Cy7–CD39 (25 μg/ml, 25-0399-42; eBioscience, Hatfield, U.K.) for 15 min on ice. The cells were then treated with 1-Step Fix/Lyse Solution (00-5333-57; eBioscience), washed in 1× Permeabilization Buffer (00-8333-56; eBioscience), and incubated with 8 μg/ml rabbit anti-human P2X1 polyclonal Ab (APR001; Alomone Labs, Jerusalem, Israel) or a rabbit IgG Ab (X0936; Dako UK, Ely, U.K.) at room temperature for 30 min. Rabbit Abs were detected using Alexa Fluor 647–goat anti-rabbit secondary F(ab’)2 (1 μg/ml, A21246; Life Technologies) for 15 min on ice. Cells were washed prior to fixation in FluoroFix buffer (BioLegend) and were acquired immediately on a BD FACSAnCanto A flow cytometer. Using FACSDiva software, eosinophils were identified using light scatter (side scatter versus forward scatter plot) and gated onto a PE histogram to measure the expression of CD11b and CD11b activated form. The data are expressed as the cellular-specific ΔGMFI following subtraction of isotype control–treated cells.

Eosinophil adhesion assay

Cell adhesion was assessed as residual eosinophil peroxidase activity of adherent eosinophils, as previously described (32). Variations to the original protocol were introduced as follows. The 96-well microplates were coated overnight at 4°C with 100 μl BSA (50 μg/ml) dissolved in HBSS (Life Technologies). Eosinophils (3 × 10^5 cells/100 μl) were treated with 3.2 IU apyrase and incubated or not with NF449 (1 μM) for 5 min at room temperature prior to cell stimulation with IL-5 (10 ng/ml), α,β-methylene ATP (α,β-meATP; 10 μM), or vehicle. Absorbance was measured at 490 nM in a microplate reader (TECAN Infinite M200; Tecan, Reading, U.K.).

Statistical analysis

Data were analyzed with GraphPad Prism 6 software and are presented as mean ± SEM. Unless stated in the figure legend, the Student t test was performed when Gaussian distribution was observed, otherwise the Wilcoxon matched-pairs and Mann–Whitney tests were used for paired and unpaired values, respectively. For electrophysiology experiments, currents from at least three cells were averaged per donor. CD11b flow cytometry experiment significance was assessed by the ratio paired test. Significance was accepted at p < 0.05.

Results

Healthy human eosinophils express P2X1, P2X4, and P2X5 receptor transcripts

The expression of P2X receptor mRNAs in human eosinophils was determined by RT-PCR. Transcripts for P2X1, P2X4, and P2X5 were detected in all samples.

Intracellular calcium concentration measurements in platelet suspensions

Preparation of platelet-rich plasma and washed platelet suspensions, as well as ratiometric fluorescence measurements of intracellular Ca^{2+} from stirred suspensions of Fura-2–loaded human platelets, was conducted as previously described (31). Experiments were conducted at 37°C in a Cairn spectrophluorometer system (Cairn Research, Faversham, U.K.), and intracellular calcium concentration ([Ca^{2+}]_i) was calculated using a dissociation constant for Ca^{2+} of 224 nM, according to Rolf et al. (28). Platelet count was measured using a z2 Coulter Counter (Beckman Coulter, High Wycombe, U.K.) and adjusted to yield an equal platelet density in paired healthy and asthmatic samples. Two-way ANOVA followed by a Bonferroni posttest were used for same-day paired healthy/asthmatic platelet [Ca^{2+}], measurements (no significant difference was observed between the groups).

CD11b/CD11b activated form cell surface expression measurements

Eosinophils (10^5 cells/100 μl buffer W) were subjected to various agonist stimulations for 2 h at 37°C in a humidified atmosphere of 5% CO2. The cells were washed and incubated with PE–anti-human CD11b (clone ICRF44, 15 μg/ml), PE–anti-human CD11b activated form (activation-sensitive anti-CD11b mAb clone CBM15/5, 20 μg/ml), or their respective PE isotype-control Ab (clone MOPC-21) at matching concentrations (Bio-Legend) in 100 μl buffer W for 15 min at room temperature. Finally, the cells were washed and resuspended in 400 μl FluoroFix Buffer, and ≥10,000 eosinophil events were acquired immediately on a BD FACSAnCanto A flow cytometer. Using FACSDiva software, eosinophils were identified using light scatter (side scatter versus forward scatter plot) and gated onto a PE histogram to measure the expression of CD11b and CD11b activated form. The data are expressed as the cellular-specific ΔGMFI following subtraction of isotype control–treated cells.

Statistical analysis

Data were analyzed with GraphPad Prism 6 software and are presented as mean ± SEM. Unless stated in the figure legend, the Student t test was performed when Gaussian distribution was observed, otherwise the Wilcoxon matched-pairs and Mann–Whitney tests were used for paired and unpaired values, respectively. For electrophysiology experiments, currents from at least three cells were averaged per donor. CD11b flow cytometry experiment significance was assessed by the ratio paired test. Significance was accepted at p < 0.05.

Results

Healthy human eosinophils express P2X1, P2X4, and P2X5 receptor transcripts

The expression of P2X receptor mRNAs in human eosinophils was determined by RT-PCR. Transcripts for P2X1, P2X4, and P2X5 were detected in all samples.

Intracellular calcium concentration measurements in platelet suspensions

Preparation of platelet-rich plasma and washed platelet suspensions, as well as ratiometric fluorescence measurements of intracellular Ca^{2+} from stirred suspensions of Fura-2–loaded human platelets, was conducted as previously described (31). Experiments were conducted at 37°C in a Cairn spectrophluorometer system (Cairn Research, Faversham, U.K.), and intracellular calcium concentration ([Ca^{2+}]_i) was calculated using a dissociation constant for Ca^{2+} of 224 nM, according to Rolf et al. (28). Platelet count was measured using a z2 Coulter Counter (Beckman Coulter, High Wycombe, U.K.) and adjusted to yield an equal platelet density in paired healthy and asthmatic samples. Two-way ANOVA followed by a Bonferroni posttest were used for same-day paired healthy/asthmatic platelet [Ca^{2+}], measurements (no significant difference was observed between the groups).

CD11b/CD11b activated form cell surface expression measurements

Eosinophils (10^5 cells/100 μl buffer W) were subjected to various agonist stimulations for 2 h at 37°C in a humidified atmosphere of 5% CO2. The cells were washed and incubated with PE–anti-human CD11b (clone ICRF44, 15 μg/ml), PE–anti-human CD11b activated form (activation-sensitive anti-CD11b mAb clone CBM15/5, 20 μg/ml), or their respective PE isotype-control Ab (clone MOPC-21) at matching concentrations (Bio-Legend) in 100 μl buffer W for 15 min at room temperature. Finally, the cells were washed and resuspended in 400 μl FluoroFix Buffer, and ≥10,000 eosinophil events were acquired immediately on a BD FACSAnCanto A flow cytometer. Using FACSDiva software, eosinophils were identified using light scatter (side scatter versus forward scatter plot) and gated onto a PE histogram to measure the expression of CD11b and CD11b activated form. The data are expressed as the cellular-specific ΔGMFI following subtraction of isotype control–treated cells.

Eosinophil adhesion assay

Cell adhesion was assessed as residual eosinophil peroxidase activity of adherent eosinophils, as previously described (32). Variations to the original protocol were introduced as follows. The 96-well microplates were coated overnight at 4°C with 100 μl BSA (50 μg/ml) dissolved in HBSS (Life Technologies). Eosinophils (3 × 10^5 cells/100 μl) were treated with 3.2 IU apyrase and incubated or not with NF449 (1 μM) for 5 min at room temperature prior to cell stimulation with IL-5 (10 ng/ml), α,β-methylene ATP (α,β-meATP; 10 μM), or vehicle. Absorbance was measured at 490 nM in a microplate reader (TECAN Infinite M200; Tecan, Reading, U.K.).

Statistical analysis

Data were analyzed with GraphPad Prism 6 software and are presented as mean ± SEM. Unless stated in the figure legend, the Student t test was performed when Gaussian distribution was observed, otherwise the Wilcoxon matched-pairs and Mann–Whitney tests were used for paired and unpaired values, respectively. For electrophysiology experiments, currents from at least three cells were averaged per donor. CD11b flow cytometry experiment significance was assessed by the ratio paired t test. Significance was accepted at p < 0.05.
receptors were expressed in eosinophils from healthy and asthmatic donors (n = 3, Fig. 1A). P2X2, P2X3, P2X6, and P2X7 receptors were below the limit of detection. Real-time PCR, which was performed to more accurately assess the level of expression of the different P2X subtypes, confirmed the presence of P2X1, P2X4, and P2X5 mRNAs in healthy eosinophils (P2X5 n = 3, Fig. 1B), whereas the remaining P2X subtypes were not confidently detected.

Presence of P2X1 receptor currents in human eosinophils
We used an electrophysiological approach to characterize eosinophil P2X receptors. The different receptor subtypes can be discriminated based on their time course and sensitivity to agonists and antagonists. ATP (100 μM) evoked rapidly activating and desensitizing inward currents (peak current 75 ± 19 pA/pF, time constants of rise of 7 ± 1 ms and monophasic decay 57 ± 4 ms, n = 3) in healthy human eosinophils (Fig. 2A). These characteristics are typical of recombinant P2X1 and P2X3 homeric receptors (5). Because homomeric P2X4 (5) and P2X5 (33), as well as heteromeric P2X1/P2X4 (8) and P2X1/P2X5 (7), receptors exhibit slow desensitization, we can exclude their functional presence in eosinophils. The potent and selective P2X1 receptor antagonist NF449 (1 μM) (34) abolished the ATP-evoked current (3 ± 1 pA/pF, time constants of rise of 7 ± 1 ms and monophasic decay 57 ± 4 ms, n = 6, p = 0.0313) (Fig. 2A, 2C). Therefore, this finding, together with the absence of detectable P2X3 transcripts (Fig. 1A and qPCR observations), rules out the expression of functional P2X3 receptor in eosinophils. The nonhydrolyzable P2X2 receptor agonist α,β-meATP (10 μM) also induced a fast transient current in eosinophils (18 ± 3 pA/pF; time constants of rise of 99 ± 17 ms and decay 58 ± 10 ms, n = 3), which was essentially abolished by 1 μM NF449 (3 ± 2 pA/pF, n = 8, p = 0.0078) (Fig. 2B, 2D). Together with the RT-PCR/qPCR data, these results indicate that human eosinophils from healthy subjects express functional P2X1 receptors.

P2X1 receptor currents are reduced in eosinophils from asthmatic donors
P2X1 receptor currents, induced by 10 μM α,β-meATP, were 3.2-fold smaller in eosinophils from asthmatic donors (8 ± 2 pA/pF, n = 12) compared with healthy donors (27 ± 5 pA/pF, n = 13, p < 0.0001) (Fig. 3A, 3B), without a change in whole-cell capacitance (1.8 ± 0.1 and 2.0 ± 0.2 pF, respectively). To determine whether the reduction in α,β-meATP-evoked current amplitude resulted from a decrease in P2X1 expression level in asthmatic eosinophils, we measured P2X1 transcript and protein levels. Similar levels of P2X1 (P2X4 and P2X5) mRNA were detected for eosinophils from healthy and asthmatic donors using RT-PCR and qPCR (Fig. 1). In addition, no change in eosinophil P2X1 total protein level was observed by Western blot or flow cytometry (Fig. 4) performed either directly on eosinophils stained in whole blood (ΔGMFI 1253 ± 119 for healthy, n = 8; 1170 ± 104 for asthmatic, n = 12) or on purified eosinophils (n = 4 for each, Supplemental Fig. 1A, 1B), therefore ruling out the possibility of a defective translational event. This suggests that other factors, such as post-translational events, regulatory proteins, or desensitization could be modulating P2X1 activity in asthmatic eosinophils. Interestingly, P2X2 receptor agonist α,β-meATP (10 μM) induced similar rapid transient increases in intracellular Ca2+ in platelets from asthmatic and healthy donors, suggesting that the intrinsic properties of P2X1 receptor in platelets are unaffected in asthma and that the loss of eosinophil P2X1 responses in asthmatic patients is not widespread among all blood cells (Fig. 5).

High concentrations of apyrase increase asthmatic eosinophil P2X1 receptor currents
The reduced P2X1 receptor currents observed in asthmatic eosinophils could result from increased receptor desensitization due to the presence of extracellular nucleotides. To test this hypothesis, eosinophils from asthmatic donors were treated with a substantial (10 IU/ml) or a standard (0.32 IU/ml) dose of the soluble nucleotidase apyrase before the recordings took place. The high apyrase concentration rescued P2X1 receptor activity in asthmatic eosinophils (α,β-meATP [10 μM]-induced currents of 17 ± 5 pA/pF for 10 IU/ml and 11 ± 3 pA/pF for 0.32 IU/ml of apyrase, n = 6, p = 0.0313) (Fig. 3C), but it had no effect on healthy eosinophils (12 ± 3 pA/pF for 10 IU/ml and 17 ± 6 pA/pF for 0.32 IU/ml, n = 3). These data suggest the desensitization of P2X1 receptor by extracellular nucleotides.
**P2X1 receptor activation upregulates the expression of CD11b activated form in healthy eosinophils**

P2X1 receptor regulates CD11b (from the integrin complex α<sub>5</sub>β<sub>2</sub>) expression in many hematopoietic cell types (23, 35, 36). αβ-meATP (10 μM) caused an increase in CD11b activated form (37) on eosinophils from healthy donors (143 ± 21% of control response, donors n = 13, p = 0.0409) but not from asthmatic donors (108 ± 11% of control response, n = 10) (Fig. 6A, 6E, 6F). ATP (100 μM) induced similar responses on eosinophils from healthy (121 ± 20%, n = 13) and asthmatic (112 ± 17%, n = 10) donors (significance was not reached because of interdonor variability) (Fig. 6B, 6E, 6F). PAF (1 μM) and eotaxin (100 ng/ml) increased eosinophil CD11b activates form in healthy (84 ± 45%, n = 10, p = 0.0311) and 170 ± 12%, n = 13, p = 0.0004, respectively) and asthmatic (133 ± 14%, n = 10, p = 0.0337 and 146 ± 20%, n = 10, p = 0.0357, respectively) donors (Fig. 6C–F). The basal levels of expression of CD11b activated form were similar in healthy and asthmatic eosinophils (2111 ± 275 ΔGMFI, n = 13 and 2341 ± 289 ΔGMFI, n = 10, respectively) (Fig. 6G).

PAF and eotaxin also increased overall eosinophil CD11b cell surface expression in healthy (121 ± 4%, n = 7, p = 0.0009 and 116 ± 7%, n = 8, p = 0.0423, respectively) and asthmatic (129 ± 4%, n = 12, p < 0.0001 and 109 ± 3%, n = 12, p = 0.0068, respectively) donors. However, αβ-meATP or ATP had no effect (105 ± 5%, n = 8 and 97 ± 4%, n = 7, respectively, for healthy) (99 ± 3%, n = 12 and 93 ± 2%, n = 12, respectively, p = 0.0078, for asthmatics). Basal levels of CD11b expression were similar in healthy and asthmatic eosinophils (10427 ± 1037 ΔGMFI, n = 8 and 10926 ± 742 ΔGMFI, n = 12, respectively). These results show that P2X1 receptor activation upregulates the expression of CD11b activated form in healthy eosinophils.

**P2X1 receptor activation increases healthy eosinophil adhesive properties**

Because α<sub>5</sub>β<sub>2</sub> integrin mediates eosinophil adhesion to ICAM-1 and BSA (32), the contribution of the P2X1 receptor to eosinophil adhesion on BSA-coated plates. Cell adhesion was assessed as residual peroxidase activity of attached eosinophils, as previously described (32). αβ-meATP (10 μM) increased healthy (180 ± 23% of control response, n = 6, p = 0.0060), but not asthmatic (123 ± 13% of control, n = 6), eosinophil adhesion (Fig. 7). NF449 (1 μM) inhibited αβ-meATP-mediated healthy eosinophil adhesion (121 ± 14% of control, n = 6, p = 0.0320). It also significantly reduced the adhesion of asthmatic eosinophils treated with αβ-meATP (89 ± 12% of control, n = 6, p = 0.0201). The latter can be explained by the fact that P2X1 currents are dramatically decreased in eosinophils from asthmatic donors (Fig. 3B). Alone, NF449 did not alter eosinophil adhesion (135 ± 20% and 109 ± 17% of control for healthy [n = 6] and asthmatic [n = 6], respectively). IL-5 (10 ng/ml) similarly potentiated healthy and asthmatic eosinophil adhesion (189 ± 23% and 171 ± 22% of control for healthy [n = 6, p = 0.0019] and asthmatic [n = 6, p = 0.0128], respectively). There was no difference in the basal adhesion levels of healthy and asthmatic eosinophils (the raw percentage of cell adhesion was 10 ± 1% and 10 ± 0% for healthy [n = 6] and asthmatic [n = 6], respectively). These data suggest that P2X1 receptor regulates healthy eosinophil α<sub>5</sub>β<sub>2</sub> integrin–dependent adhesion.

**FIGURE 6.** P2X1 receptor increases the expression of healthy eosinophil CD11b activated form. Effect of αβ-meATP (10 μM) (A), ATP (100 μM) (B), PAF (1 μM) (C), and eotaxin (100 ng/ml) (D) on CD11b activated form expression in healthy and asthmatic eosinophils. Summary of the effect of PAF (1 μM), eotaxin (100 ng/ml), ATP (100 μM), and αβ-meATP (10 μM) on eosinophil CD11b activated form expression level in healthy (E) and asthmatic (F) donors. (G) Basal level of CD11b activated form in healthy and asthmatic eosinophils. n = number of donors. *p < 0.05, ***p < 0.005, ns, not significant.
Discussion

In addition to their antiparasitic activity and involvement in allergic reactions, eosinophils are associated with the development of airway diseases. A range of ligands regulates eosinophil function (3); among these, extracellular nucleotides are important. Extracellular nucleotides, acting at P2 receptors, control many cell functions, including platelet activation (38) and modulation of inflammation (39). In the current study, we show that healthy human eosinophils express functional P2X1 receptors, P2X1 activation leads to increased eosinophil αβ2 integrin–dependent adhesion, and P2X1 receptor responses are reduced in asthmatic eosinophils.

We demonstrated the expression of P2X1, P2X4, and P2X5 receptor subtype transcripts in healthy donor eosinophils, as reported by other groups (18, 19). In agreement with Mohanty et al. (19), we could not detect P2X7 receptor mRNA in eosinophils, although it was reported by Ferrari et al. (18). This difference could be due to the high purity of our eosinophil preparation (99.4 ± 0.0%) containing a negligible proportion of other hematopoietic cells, including platelets (Supplemental Fig. 2). The original full-length P2X7 receptor gene is composed of 13 exons and a plethora of splice variants, and polymorphisms have been identified (40). Our sets of primers amplify sequences ranging from exons 6 to 11 (27) and from exons 10 to 13. They target the known functional P2X7 splice isoforms, as well as nonfunctional variants (40), therefore ruling out the presence of functional P2X7 receptors in human eosinophils.

Although P2X5 receptor transcripts were the most abundant in human eosinophils (~35-fold more than P2X1), no P2X5 current feature was observed (33). This is consistent with P2X5 receptor splice variant in humans lacking the pore-forming domain of the channel (41). The absence of P2X4 currents (42) could be explained by the low levels of receptor mRNA (~10-fold less than P2X1). The presence of functional P2X2/P2X4 (8) and P2X1/P2X5 (7) heteromeric receptors is also unlikely because they would display current characteristics different from those observed in human eosinophils. The currents elicited by the extracellular nucleotide ATP and its structural analog α,β-metATP exhibited kinetics (43) and sensitivity to NF449 (34) that are characteristic of the P2X1 receptor, confirming the functional presence of this receptor in human eosinophils. While our manuscript was under revision, a study by Alberto et al. (44), describing the presence of P2X1-like receptor currents in rat eosinophils, was published, further supporting our findings. This functional homomeric P2X1 receptor phenotype is not a trait unique to eosinophils; it is also observed in platelets (45) and neutrophils (46). However, it seems that divergence exists within the hematopoietic lineage because mast cells (27) and macrophages (47) exhibit additional P2X receptor subtype activities (i.e., P2X4 and P2X7).

P2X1 currents were 3.2-fold smaller in eosinophils from asthmatic donors compared with healthy donors. The diminished currents are unlikely to be due to defective transcriptional and/or translation event(s) because P2X1 transcript and protein expression levels were similar in cells from asthmatic and healthy donors; although the possibility that defective P2X1 receptors could originate from a compromised intracellular pool in asthmatics cannot be ruled out.

In contrast, P2X1 activity was similar in healthy and asthmatic platelets, indicating that P2X1–decreased activity in asthmatic eosinophils has a degree of specificity. Eosinophils in asthma are presumed to be exposed to increased concentrations of growth factors, such as IL-5, which prime eosinophils for enhanced function (48). However, IL-5 did not induce a reduction in P2X1 current amplitude, suggesting that other causal events are responsible for altering P2X1 responses in eosinophilic asthma (Supplemental Fig. 3).

Under inflammatory conditions, cells can release nucleotides into the extracellular environment (12, 14, 16, 17, 49), desensitizing neighboring P2 receptors and causing aberrant regulation of P2 receptor functions (50). In asthmatic eosinophils, P2X1 activity was rescued when the cells were treated with high concentrations of apyrase. This is consistent with asthmatic eosinophil P2X1 receptors being desensitized as a result of exposure to increased amounts of extracellular ATP. An increase in extracellular nucleotides could be derived from eosinophils through an autocrine pathway (12) and/or neighboring cells (15). We found no difference in the basal ATP release level within the bulk extracellular medium of eosinophils from healthy and asthmatic donors. However, ATP could be released in extracellular subcompartments where it is degraded rapidly by locally expressed ectonucleotidases, as shown by Joseph et al. (51). Alternatively, an increase in extracellular ATP could be the result of a lower extracellular nucleotide clearance by ectonucleotidases. Interestingly, a recent report by Wang et al. (52) showed the downregulation of the transcripts for the ectonucleotidase CD39 in PBMCs from asthmatic patients, suggesting that CD39 mRNA deficiency could contribute to the asthmatic phenotype. Although we did not observe any change in the expression level of CD39 protein between healthy and asthmatic donors, however, ATP could be released in extracellular subcompartments where it is degraded rapidly by locally expressed ectonucleotidases, as shown by Joseph et al. (51). Alternatively, an increase in extracellular ATP could be the result of a lower extracellular nucleotide clearance by ectonucleotidases. Interestingly, a recent report by Wang et al. (52) showed the downregulation of the transcripts for the ectonucleotidase CD39 in PBMCs from asthmatic patients, suggesting that CD39 mRNA deficiency could contribute to the asthmatic phenotype. Although we did not observe any change in the expression level of CD39 protein between healthy and asthmatic eosinophils (Supplemental Fig. 1C–E), we cannot exclude the involvement of a regulatory protein malfunction and/or other ectonucleotidases.

Idzko et al. showed that the P2X receptor agonist α,β-metATP, at concentrations up to 1 mM, induced Ca2+ transients, chemotaxis, ROS production, actin polymerization, IL-8 release, and increased CD11b cell surface expression in healthy human eosinophils (22, 23). In our study, using a submaximal concentration of...
α,β-meATP (53), ROS generation, cell shape change, upregulation of CD11b expression, and chemotaxis were not observed in healthy or asthmatic eosinophils (data not shown).

Like in human eosinophils, P2X1 is the only functional P2X receptor subtype in neutrophils (46) and platelets (54). Although P2X1 currents are also relatively small in these cells (<100 pA/pF) (46, 54), their physiological effects can be rather extensive. For example, P2X1 receptors promote neutrophil chemotaxis (46) and protect against endotoxemia by dampening neutrophil activation (35). In platelets, P2X1 amplifies the responses (e.g., aggregation and dense granule secretion) of many ligands (e.g., collagen, thrombin, and ADP) (55, 56). Platelet P2X1 receptor deficiency or inhibition dramatically reduces aggregation mediated by low concentrations of collagen (0.5–1.25 μg/ml) (54). However, α,β-meATP or NF449 had no effect on PAF- or eotaxin-induced eosinophil respiratory burst and shape change, suggesting that the physiological role of P2X1 in eosinophils is not to fine-tune the effect of these natural eosinophil ligands (data not shown).

P2X1 receptor regulates integrin activity in hematopoietic cells, such as platelets (54), neutrophils, and monocytes (35), whereas in healthy eosinophils, P2X1 activation upregulates CD11b expression (23). The integrin αMβ2 is involved in eosinophil adherence-dependent function, including transmigration and degranulation (32, 37, 57). Our study showed that, although P2X1 activation had no effect on the overall expression of CD11b, it increased the expression of the activated state of the integrin in healthy, but not asthmatic, eosinophils. Zhu et al. (32) reported that eosinophils can bind to receptors whose activation leads to an increased eosinophil a activates ion channels. This delicate balance seems to be lost in asthmatic patients; therefore, the rehabilitation of P2X1 receptor activation is an important goal in asthma therapy.

In summary, healthy human eosinophils express functional P2X1 receptors whose activation leads to an increased eosinophil αMβ2 integrin–dependence. This could represent a mechanism for retention of eosinophils in a tissue compartment. Because P2X1 receptors are sensitive to low ATP concentrations (e.g., due to constitutive release from neighboring cells), and consequently desensitization (5), a subtle equilibrium needs to be reached between the release of ATP and the activity of the local ectonucleotidases. This delicate balance seems to be lost in asthmatic patients; therefore, the rehabilitation of P2X1 receptor activity could be a new therapeutic target for asthma.

Acknowledgments
We thank all of our blood volunteers.

Disclosures
The authors have no financial conflicts of interest.

References
16. Esther, C. R., Jr., N. E. Alexis, M. L. Claus, E. R. Lazaroni, S. H. Donaldson, C. M. Roibeiro, C. G. Morel, A. D. Davis, and C. R. Breer. 2008. Extracellular ATP is a ligand for BSA and ICAM-1 in an αMβ2-dependent manner. Following their eosinophil-BSA–adhesion protocol, we found that P2X1 receptor activation caused an increase in healthy (but not asthmatic) eosinophil adhesion, revealing the contribution of P2X1 to eosinophil αMβ2 integrin–dependence. This could represent a mechanism for retention of eosinophils in a tissue compartment. Because P2X1 receptors are sensitive to low ATP concentrations (e.g., due to constitutive release from neighboring cells), and consequently desensitization (5), a subtle equilibrium needs to be reached between the release of ATP and the activity of the local ectonucleotidases. This delicate balance seems to be lost in asthmatic patients; therefore, the rehabilitation of P2X1 receptor activity could be a new therapeutic target for asthma.

In summary, healthy human eosinophils express functional P2X1 receptors whose activation leads to an increased eosinophil αMβ2-dependent adhesion that is absent in asthmatic eosinophils. P2X1 responses are constitutively reduced in asthmatic eosinophils compared with healthy eosinophils, likely as the result of an increase in extracellular nucleotide concentration. Our work suggests that P2X1 receptor regulates eosinophil homeostasis because the receptor function is compromised in asthmatic eosinophils.
### Supplemental Table 1. List of primers used for classical RT-PCR and qPCR

<table>
<thead>
<tr>
<th>Genes (human)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-TGGTGGGCCATGGGTGCTCAG-3'</td>
<td>5'-GTCCCGGCGCCAGCCAGGTCCAG-3'</td>
</tr>
<tr>
<td>P2X1</td>
<td>5'-CGTCATCGGGGTGGGTGTTTCTCTA-3'</td>
<td>5'-AGGGCCGCGGATGCTGCA-3'</td>
</tr>
<tr>
<td>P2X2</td>
<td>5'-GGGCCCCGAGAGTCCATCATC-3'</td>
<td>5'-GCGAGGGAGGTCCAGTCACAGTCC-3'</td>
</tr>
<tr>
<td>P2X3</td>
<td>5'-ACTGGGCGCTGGTGAAGTCA-3'</td>
<td>5'-CAGGTCGAAGCGGATGCAAAAG-3'</td>
</tr>
<tr>
<td>P2X4</td>
<td>5'-CGGCACCCACAGCAACGGATCT-3'</td>
<td>5'-TGTATCGAGGCGGGCGGAAGGTA-3'</td>
</tr>
<tr>
<td>P2X5</td>
<td>5'-GGGCCCAAGAACCACCACACTGC-3'</td>
<td>5'-CCTCGGGCCTCGGGAAGTCTCT-3'</td>
</tr>
<tr>
<td>P2X6</td>
<td>5'-AGCCCCCTACTGTCCGGTGTCC-3'</td>
<td>5'-GCCTTGGCCTCTCCTACATTGTC-3'</td>
</tr>
<tr>
<td>P2X7</td>
<td>5'-CCGCGACACAATCACACGAG-3'</td>
<td>5'-GGCCAGACCAGAGTAGGAGAG-3'</td>
</tr>
</tbody>
</table>

1. β-actin gene was used as reference gene for the classical RT-PCR. The reactions consisted of 40 cycles at 94°C for 45 s, 55°C (P2X2, 5 & 7) or 58°C (P2X1, 3, 4 & 6) for 45 s, and at 72°C for 60 s. For analysis, all RT-PCR products were run on 1.5% agarose gel containing ethidium bromide.

<table>
<thead>
<tr>
<th>Genes (human)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-TCCTATGTGGGGCGAG-3'</td>
<td>5'-ATGGCTGGGTTGAAG-3'</td>
</tr>
<tr>
<td>PPIB (cyclophilin B)</td>
<td>5'-CGTCATCGGGGTGGGTGTTTCTCTA-3'</td>
<td>5'-ACCCAAATCTTTCTCTCTG-3'</td>
</tr>
<tr>
<td>RPL13A1</td>
<td>5'-CTGCTTTCTTTCTGTGCTG-3'</td>
<td>5'-AGCCAAATCTTTTCTCTCAG-3'</td>
</tr>
<tr>
<td>P2X1</td>
<td>5'-CTGGTGGAGGAGGTGAATG-3'</td>
<td>5'-AAGTGAAGCGGATGGAGGAG-3'</td>
</tr>
<tr>
<td>P2X2</td>
<td>5'-CATCGGGGTCCATTATCAAC-3'</td>
<td>5'-CAGTCGACAGGAAGGAG-3'</td>
</tr>
<tr>
<td>P2X3</td>
<td>5'-GACCTTCTCTTGGCACCATTAC-3'</td>
<td>5'-CCTGCCATTTCTCAAGCAG-3'</td>
</tr>
<tr>
<td>P2X4</td>
<td>5'-GGAGAACGAGGACACAG-3'</td>
<td>5'-CCTCCCCAACAAGATGATG-3'</td>
</tr>
<tr>
<td>P2X5</td>
<td>5'-TGGTCGTATGGGTGTACAG-3'</td>
<td>5'-TGCTTCATTCTACAGCAG-3'</td>
</tr>
<tr>
<td>P2X6</td>
<td>5'-CCGCGACACAATCACACGAG-3'</td>
<td>5'-GAAGGTGACTGTGTGTTGATG-3'</td>
</tr>
<tr>
<td>P2X7</td>
<td>5'-TACCATCGGCTCAACCCTCTC-3'</td>
<td>5'-GCAGGCTTTGGGAAGTCCTTG-3'</td>
</tr>
</tbody>
</table>

2. β-actin, PPIB (cyclophilin B) and RPL13A1 genes were used as reference genes for the qPCR. The reactions consisted of 38 cycles at 95°C for 15 s, 60°C for 15 s, and at 72°C for 30 s.
Assessment of human eosinophil P2X1 receptor and CD39 protein expression levels by flow cytometry:

A. and B. Extracellular and intracellular (total) P2X1 receptor expression levels were measured in eosinophils and HEK293 cells stably transfected with P2X1 tagged with GFP (HEK293-P2X1) using polyclonal rabbit antibodies (4 μg/ml) raised against extracellular (P2X1-eAb) (#APR-022, Alomone labs, Jerusalem, Israel) and intracellular (P2X1-inAb) (#APR-001, Alomone labs) P2X1 receptor epitopes. All flow cytometry acquisitions were performed using a CsnT checked BD FACS Canto (Becton Dickinson, Oxford, UK) and reported as geometric mean fluorescence intensity (GMFI). Statistics were performed using the Student paired t-test.

Total P2X1 receptor protein level in human eosinophils and HEK293-P2X1 (positive control) cells were assessed following cell fixation [250 μl of intracellular fixation buffer (eBioscience, Ltd., Hatfield, UK) was added to cells (5x10⁴) and incubated for 20 min at 4°C] and permeabilisation (eBioscience, Ltd.) on ice, all in the presence of the permeabilisation buffer. Following washing, the cells were resuspended in 400 μl of Fluorfix (Biolegend UK Ltd., London, UK) buffer for flow cytometry acquisition.

Both healthy (GMFI of 10693±613 against 7513±1184 for P2X1 antibody and IgG control respectively, p=0.0238 n=4) and asthmatic (GMFI of 9558±936 versus 7747±602 for P2X-inAb and IgG control respectively, p=0.0207, n=4) eosinophils exhibited total P2X1 receptor protein expression (A). However, there was no difference in the level of total P2X1 receptor protein expression between eosinophils from healthy and asthmatic donors. In comparison, HEK293-P2X1, used here as a positive control for the expression of P2X1 receptor protein, expressed a high level of P2X1 receptor protein (GMFI of 33801±1835 versus 3132±448 for P2X1 antibody and IgG control respectively, p=0.0002, n=4) (A).

For P2X1 receptor cell surface expression measurement, 50 μl of cell suspension (5x10⁴ cells) were incubated for 15 min at 4°C in presence of human IgG (0.4 μg/ml)(Sigma-Aldrich) to block non-specific binding of primary antibodies. Cells were then incubated either with P2X1-eAb or a control rabbit IgG (#X0936, Agilent Technologies, Dako Glostrup, Denmark) at room temperature for 30 min. Cells were subsequently washed and stained with 1 μg/ml AF647–goat anti-rabbit IgG (H+L) (Fab₂ fragment) (#A21246, Life Technologies Ltd.) on ice, all in the presence of the permeabilisation buffer. Following washing, the cells were resuspended in 400μl of Fluorfix (Biolegend UK Ltd., London, UK) buffer for flow cytometry acquisition.

P2X1 protein surface expression in eosinophils from healthy (GMFI of 622±76 versus 625±63 for P2X1-eAb and IgG control respectively, n=4) and asthmatic (GMFI of 847±17 versus 858±34 for P2X1 antibody and IgG control respectively, n=4) donors was not detected (B). In comparison cell surface P2X1 receptor protein was detected in HEK239-P2X1 (GMFI of 1239±222 versus 839±126 for P2X1-eAb and IgG control respectively, p=0.0360, n=4) though greatly reduced compared to cell total expression (B).

While we expect P2X1 receptor protein expression at the eosinophil cell surface to be low due to the small amplitudes of the currents measured, flow cytometry may not be an approach sensitive enough to measure minute receptor expression levels on per cell basis. In addition, we cannot rule out that the antibody directed against the extracellular epitope itself may not be optimal for the measurement of the weakly expressed P2X1 protein.

C, D and E. Eosinophil P2X1 receptor and CD39 expression levels were measured from whole blood. See Protocol in main manuscript “Flow cytometric detection of eosinophil P2X1 total protein expression” (C).

The geometric mean fluorescence intensity (ΔGMFI) of Eosinophil Siglec-8, P2X1R and CD39 expression (or relevant controls) was calculated. Compared to FMO (Red in D) and polyclonal controls (Blue in D), Eosinophils expressed both CD39 and P2X1R (Orange in D), respectively. Total CD39 ΔGMFI (2905±284 vs 3138±325) fluorescence values were similar between healthy (n=8) and asthmatic (n=12), respectively (E).
Platelets do not bind to eosinophils in our eosinophil preparations:

To detect whether platelets were bound to eosinophils, purified eosinophils \((1\times10^5/100\ \mu l)\) were incubated with PE-conjugated mouse anti-human CD42b (clone HIP-1, BioLegend UK Ltd.) or PE-conjugated IgG1 isotype control (clone MOPC-21, BioLegend UK Ltd) at the same final concentration \((0.125\mu g/ml)\) in PBS (+2% AB serum, Invitrogen) for 30 minutes at room temperature. Subsequently, 400\(\mu l\) of PBS were added and at least 10,000 eosinophil events were acquired immediately on a BD FACSCanto flow cytometer using FACSDiva software. As a positive control, we collected autologous platelet rich plasma (PRP)\(^1\) and stained PRP \((3\times10^6\) platelets, volume adjusted to 100\(\mu l\) with PBS) using the same protocol as described above (at least 10,000 platelet events were acquired). We also verified that platelets can bind to eosinophils using our experimental approach by co-incubating the purified eosinophils and PRP \((1\times10^5\) and \(3\times10^6\) cells in 100\(\mu l\) for eosinophils and platelets respectively) and 10,000 eosinophil events were acquired on a BD FACSCanto flow cytometer. For both cell populations, we measured cell associated fluorescence in the PE channel. The data shown are expressed as % of cells positive for CD42b following subtraction of the isotype control treated cells. Statistics were performed using the Mann-Whitney test.

Jawien et al. have previously reported the binding of platelets to eosinophils, therefore raising the possibility of cross-contamination of our preparation of eosinophil RNA with platelet RNA\(^2,3\). To determine if our eosinophil preparations were contaminated with platelets, the cell suspensions were incubated with the platelet-selective antibody anti-CD42b and analysed by flow cytometry. Platelets were not detected in the eosinophil preparations from healthy or asthmatic donors \([1.1\pm0.5\%\) and \(0.4\pm0.3\%\) of cells were CD42b-positively labelled for healthy \((n=7)\) and asthmatic \((n=14)\), respectively\]. In contrast, high level of CD42b expression was observed in platelet rich plasma \([98\pm1\%\) and \(93\pm3\%\) of cells were CD42b-positively labelled for healthy \((n=7)\) and asthmatic \((n=14)\), respectively\]. In accordance with Jawien et al., the co-incubation of eosinophils and PRP resulted in the binding of platelets to eosinophils \([57\pm6\%\) and \(50\pm2\%\) of eosinophils were CD42b-positively labelled for healthy \((n=7)\) and asthmatic \((n=14)\), respectively\]. These data suggest that our eosinophil preparations are not contaminated with platelets.

IL-5 treatment has no effect on eosinophil P2X1 receptor currents:

As would be expected\(^1\), the asthmatic donors used in this study had a mild eosinophilia (0.49±0.12×10^6 cells/ml, n=22, compared to 0.14±0.03×10^6 cells/ml in healthy donors, n=32, p=0.0002). This is thought to be due to excess production of IL-5 and other eosinophil growth factors. We therefore tested whether the reduced current in eosinophils from asthmatic donors could be reproduced \textit{in vitro} in eosinophils from healthy subjects by treating them with IL-5. Eosinophils from healthy donors were plated into 24 well plates (10^6 cells/ml) and treated or not with IL-5 (1 nM) for 0, 2 or 18h at 37°C in a humidified atmosphere of 5% CO\(_2\) before recordings. IL-5 had no effect on 10 μM α,β-meATP-induced P2X1 receptor currents after 2h (98±47% for control and 82±35% for IL-5 of response at time “0”) or 18 h treatment (22±11% for control and 71±39% for IL-5) (at time “0”, 100±22%),(n=3). The survival of IL-5-treated (97±2%) or non-treated eosinophils (97±2%, n=3) remained unaffected. Friedman test ANOVA followed by Dunn’s multiple comparisons test were used and significance was accepted at p<0.05.