



Effects of IL-4 and IL-13 on adenosine receptor expression and responsiveness of the human mast cell line 1

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Abstract

Background: Inhalation of adenosine-5'-monophosphate (AMP) causes bronchoconstriction in asthma but not in healthy subjects. Bronchoconstriction upon AMP inhalation is thought to occur by histamine release and subsequent binding to receptors on airway smooth muscle cells.

Methods: To explain enhanced sensitivity to AMP in asthma, mast cell expression of the adenosine A_{2A} and A_{2B} receptors and histamine release were measured after incubation of human mast cell line 1 (HMC-1) cells with AMP and the non-specific adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) for 1.5 and 6 h. To establish a Thelper-2 environment resembling the asthma phenotype, HMC-1 cells were additionally cultured with IL-4 and IL-13 alone or stimulated with the combination of both cytokines and AMP and NECA. To study effects of prolonged presence of the inflammatory environment, the cells were pre-incubated overnight (18 h) with IL-4 and IL-13 and additionally stimulated with AMP and NECA for 1.5 or 6 h.

Results: AMP and NECA hardly affected adenosine receptor expression but increased IL-8 secretion. Incubation with IL-4 and IL-13 for 6 h increased adenosine A_{2A} receptor expression and histamine secretion, but decreased IL-8 secretion. The combination of IL-4, IL-13, and AMP/NECA for 6 h increased A_{2B} receptor expression and IL-8 secretion. Overnight stimulation with IL-4, IL-13 and subsequent stimulation with AMP/NECA for 1.5 h decreased A_{2A}R expression which was accompanied by increased histamine secretion.

Conclusion: These results suggest a role for decreased A_{2A}R expression in enhanced adenosine responsiveness as observed in asthma.

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1. Introduction

Inhalation of adenosine or its more stable form adenosine-5'-monophosphate (AMP) causes bronchoconstriction in asthma but not in healthy subjects [1]. In addition, increased

concentrations of adenosine have been found at sites of inflammation and in bronchoalveolar lavage fluid of asthma patients [2]. For these reasons it is believed that adenosine and adenosine receptors have an important role in asthma [3]. Adenosine plays a role in the energy metabolism of the cell, being formed during dephosphorylation of ATP ($\rightarrow$ ADP $\rightarrow$ AMP $\rightarrow$ adenosine). It can cause the release of inflammatory mediators after binding to adenosine receptors on inflammatory cells (e.g. mast cells).

Adenosine receptors are G-protein coupled receptors and four of these receptors have been described; A_1 , A_{2A} , A_{2B} and A_3 [4]. The adenosine A_{2A} and A_{2B} receptors are G_s -protein coupled receptors [5]. After binding of adenosine they will activate adenylyl cyclase, which results in an increased production of cyclic AMP that will inhibit activation of the cell. An important difference between adenosine A_{2A} and A_{2B} receptors is the ability of adenosine A_{2B} receptors to additionally couple to G_q -proteins [6]. This then results in activation of phospholipase C which splices phosphatidylinositol-4,5 biphosphate (PIP₂) in diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG activates protein kinase C and IP₃ activates calcium channels, actions necessary for the fusion of granules of mast cells with the plasma membrane [7,8]. Binding of adenosine to the low-affinity adenosine A_{2B} receptor causes degranulation of mast cells [3,9] resulting in histamine release, which in turn may induce bronchoconstriction by binding to histamine receptors on airway smooth muscle cells. In contrast, binding of adenosine to the high-affinity A_{2A} receptor results in suppression of histamine release from mast cells [3,10–12].

IL-4 and IL-13, typical Th2 cytokines, are present in higher concentrations after allergen challenge in lungs of asthma patients compared to non-asthmatic subjects [13]. Both cytokines are produced by Th2 cells and mast cells and play an important role in allergic inflammation, such as isotype switching of B cells to IgE synthesis [14]. Both IL-4 and IL-13 can cause mast cell activation *in vivo* [15], and *in vitro* [16].

In this study we were interested in the relationship between adenosine responsiveness of mast cells and asthma. Therefore, we investigated the effects of IL-4 and IL-13 on *in vitro* mast cell function and adenosine receptor expression. Since it is difficult to obtain mast cells from asthma patients, the human mast cell line 1 (HMC-1) was used and cultured in the presence of IL-4 and IL-13. The HMC-1 mast cell line was established from peripheral blood of a patient with mast cell leukaemia [17]. These cells have a low expression of the high-affinity IgE receptor, but show expression of the adenosine A_{2A} , A_{2B} , and A_3 receptors [18,19] and express functional receptors for both IL-4 and IL-13 [20]. These features make this HMC-1 cell a suitable tool to study the effects of adenosine and Th2 cytokines on mast cells. A higher expression of the $A_{2B}R$ over $A_{2A}R$ might explain increased AMP responsiveness in asthma. We hypothesized that culturing of HMC-1 cells in a Th2 environment (IL-4, IL-13), like it is present in airways of asthma patients, would increase $A_{2B}R$ and decrease the $A_{2A}R$ expression.

2. Materials and methods

2.1. HMC-1 cell line

The leukemic mast cell line HMC-1 (a generous gift from doctor J.H. Butterfield, Mayo Clinic, Rochester MN) was maintained in suspension

culture at a density between 0.3 and 1.0×10^6 cells/ml by culturing in Iscove's Modified Dulbecco's Medium (IMDM, Gibco®, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FBS; Gibco®), 1.23 mM L-glutamine (BioWhittaker, Verviers, Belgium), 50 μ M β -mercaptoethanol (Gibco®), 5 μ g/ml apo-transferrin (Sigma, St. Louis, USA), 100 U/ml penicillin (BioWhittaker), and 100 μ g/ml streptomycin (BioWhittaker). Cells were kept under humidified atmosphere of air/CO₂ (19:1) at 37 °C.

2.2. Stimulation with AMP and NECA

To stimulate the cells via the adenosine receptors, 5'-adenosinemonophosphate (AMP) and the non-specific adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) were added to the culture medium of the cells. HMC-1 cells (1×10^6 cells/ml, $n=7$) were cultured either with AMP (0.1 μ M, Sigma), with NECA (1 μ M, Sigma), or with the combination AMP/NECA (both dissolved in IMDM, optimal concentrations obtained from previous experiments, data not shown). Cells were incubated for 1.5 or 6 h under humidified atmosphere of air/CO₂ (19:1) at 37 °C. At the end of the incubation periods cells were harvested and culture supernatants were collected by centrifugation at $500 \times g$ for 5 min at 4 °C. Cells were used for flow cytometric analysis for adenosine receptor expression and supernatants were used for histamine and IL-8 secretion.

2.3. Stimulation with IL-4 and IL-13

To investigate whether cytokines that are increased in asthma patients alter adenosine receptor expression and cell degranulation, HMC-1 cells (1×10^6 cells/ml, $n=8$) were cultured either with recombinant human IL-4 (10 ng/ml, Strathmann Biotec AG, Hamburg, Germany), with recombinant human IL-13 (20 ng/ml, PeproTech Inc, Rocky Hill, USA) or with the combination of both IL-4 and IL-13. These cytokines were added in optimal concentrations as deduced from previous experiments (data not shown). Furthermore, a combination of IL-4/IL-13 and AMP/NECA (in previously mentioned concentrations) was added in a separate experiment. The choice to stimulate HMC-1 cells with the combination of AMP and NECA instead of AMP alone was based on data from the literature in which IL-8 was the read-out parameter, produced upon activation of the $A_{2B}R$ (19;21). Cells were incubated for 1.5 or 6 h under humidified atmosphere of air/CO₂ (19:1) at 37 °C. At the end of the incubation periods cells were harvested and treated as mentioned above.

2.4. Overnight (18 h) incubation with IL-4/IL-13 (with and without AMP/NECA)

To establish an asthmatic environment for a longer time period, IL-4 and IL-13 were added overnight to the cultures before stimulation with AMP/NECA. HMC-1 cells (1×10^6 cells/ml, $n=8$) were incubated overnight (18 h) with and without the combination of IL-4/IL-13. In addition, cells were stimulated by adding the combination AMP/NECA (0.1 and 1.0 μ M respectively) for 1.5 or 6 h. Cells were incubated under humidified atmosphere of air/CO₂ (19:1) at 37 °C. At the end of the incubation periods cells were harvested and treated as mentioned above.

This experiment was repeated ($n=4$) with an extra wash step between the overnight pre-incubation with IL-4 and IL-13 and the stimulation of AMP/NECA. After centrifugation of the cell suspensions, cell pellets were resuspended in fresh HMC-1 medium with addition of the stimulants. Supernatants were used for histamine measurement only.

2.5. Pre-incubation with theophylline

To investigate whether histamine secretion following AMP/NECA stimulation could be inhibited by an antagonist for the adenosine A_{2B}

receptor, HMC-1 cells were pre-incubated for 30 min with 150 μ M theophylline (non-selective adenosine receptor antagonist with the highest affinity for $A_{2B}R$, Sigma) prior to a 6-hour stimulation with IL-4/IL-13 and/or AMP/NECA. Cells were incubated under humidified atmosphere of air/ CO_2 (19:1) at 37 °C. At the end of the incubation period cells were harvested and supernatant was stored for histamine measurements.

2.6. Flow cytometric analysis

To investigate adenosine receptor expression on HMC-1 cells, flow cytometric analysis was performed. Cells were stained with the primary antibodies rabbit-anti-canine $A_{2A}R$ (1:15, cross-reactivity with human) and rabbit-anti-human $A_{2B}R$ (1:20), both from Alpha

Diagnostic International (San Antonio, USA). The secondary antibody used was fluorescein isothiocyanate (FITC) conjugated goat-anti-rabbit (1:25, Becton Dickinson, Franklin Lakes, USA). Cells were resuspended in FACS lysing solution (Becton Dickinson). Cells (2×10^4 events) were analyzed using an Epics® Elite flow cytometer (Coulter, Fullerton, USA) and raw data analysis was performed with FlowJo (TreeStar, San Carlos, CA). The gate to calculate the MFI (mean fluorescence intensity) of FITC positive cells was set in the FITC histogram of unstained cells, the gate was set at the point where less than 1% of the cells were FITC positive, and copied to all other samples. In the first experiment an isotype control was added (normal rabbit IgG), and its histogram was similar to unstained cells. Data is presented as a percentage of control (no stimulation), which was set at 100%.

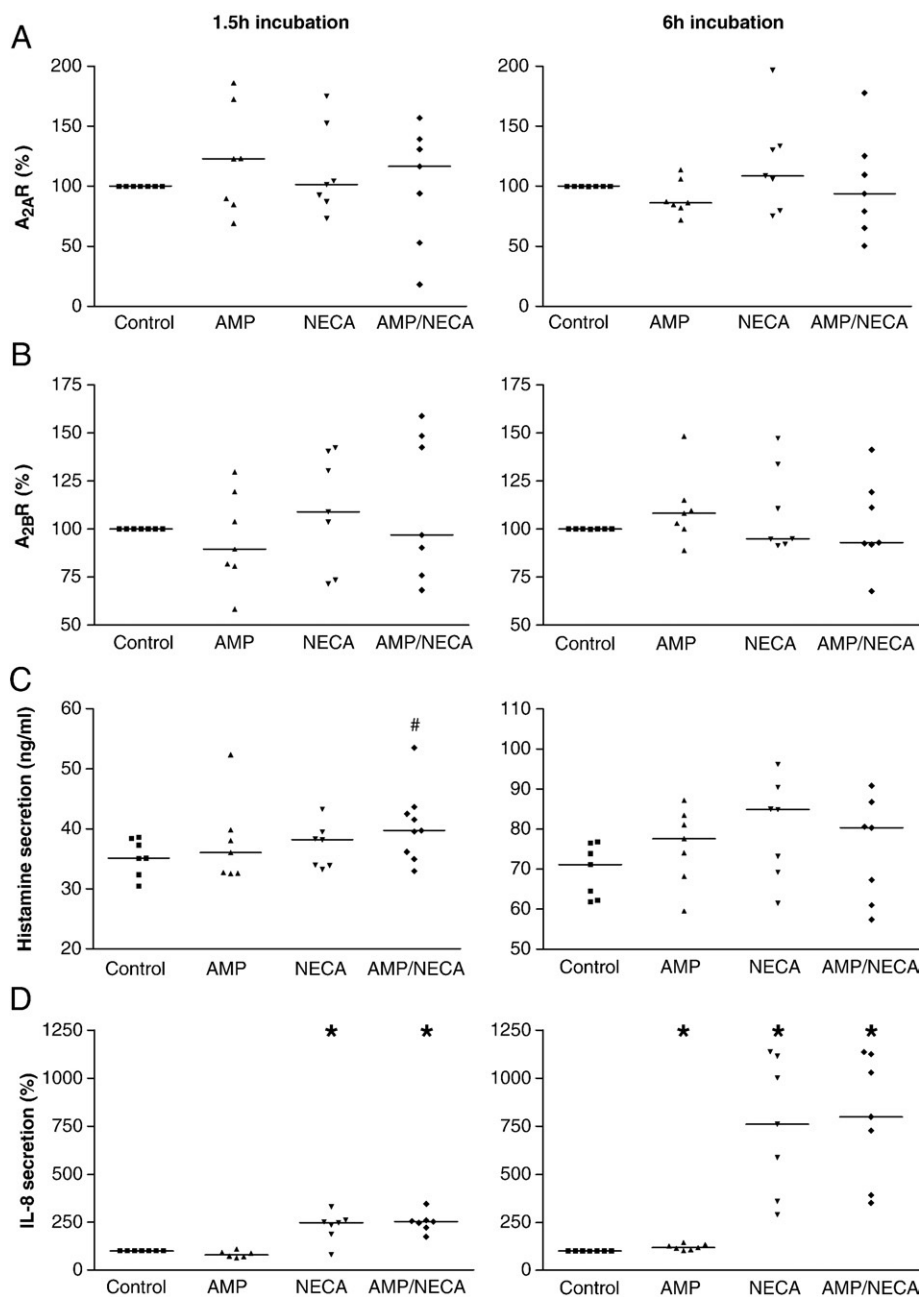


Figure 1 Adenosine A_{2A} (A) and A_{2B} (B) receptor expression, histamine release (C), and IL-8 secretion by HMC-1 cells after incubation with AMP and/or NECA for 1.5 h (left panels) and 6 h (right panels). All values (except histamine) are corrected for the control which was set at 100%. Horizontal bars represent median values. * $p < 0.05$ vs. control. # $p = 0.05$ vs. control.

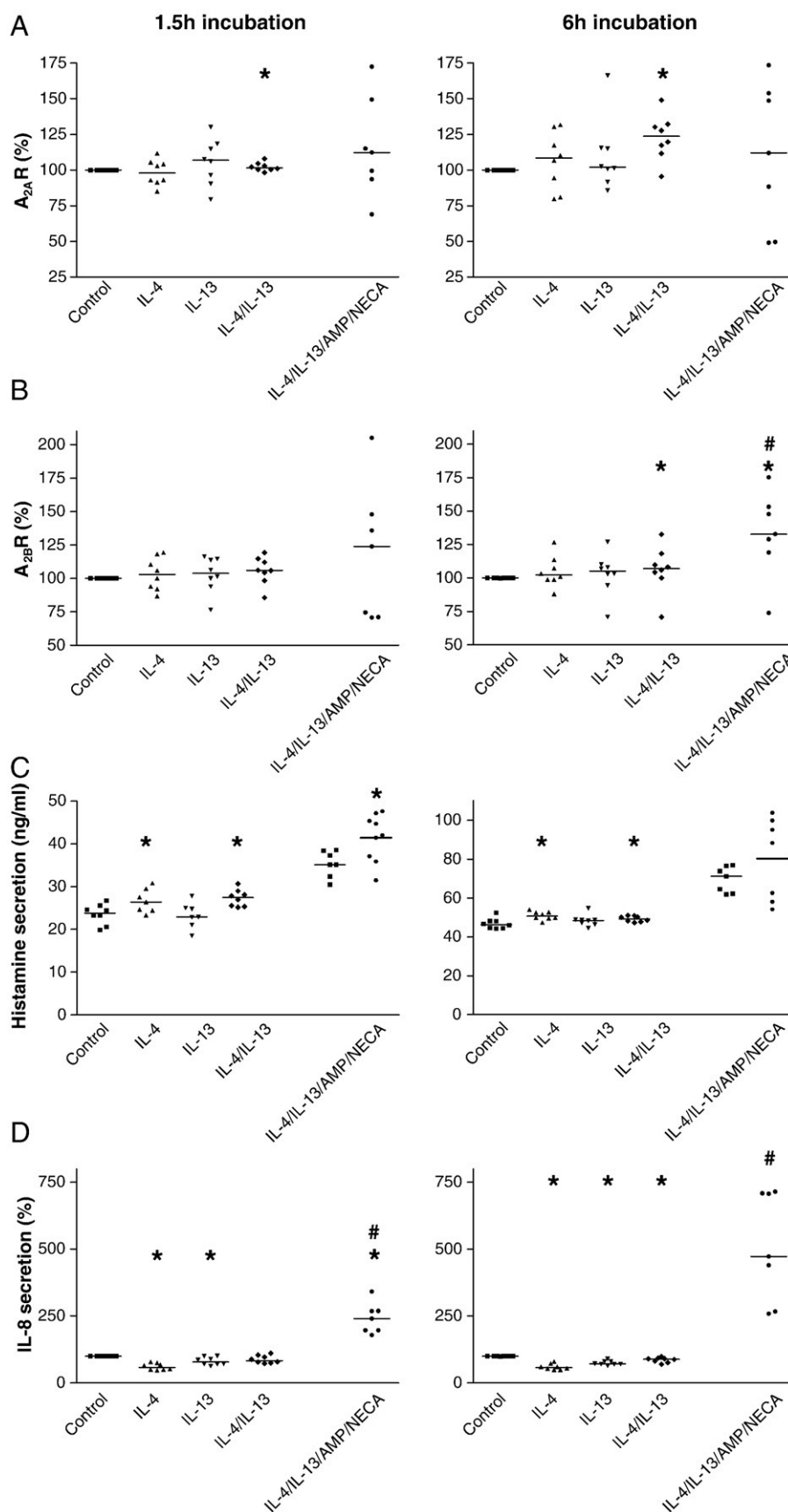


Figure 2 Adenosine A_{2A} (A) and A_{2B} (B) receptor expression, histamine release (C), and IL-8 secretion (D) after incubation of HMC-1 cells with IL-4, IL-13, IL-4/IL-13 and IL-4/IL-13/AMP/NECA for 1.5 h (left panels) and 6 h (right panels). All values (except histamine) are corrected for the control which was set at 100%. Horizontal bars represent median values. **p* < 0.05 vs. control, #*p* < 0.05 vs. IL-4/IL-13.

2.7. Histamine and IL-8 measurement

Histamine content of the culture supernatants was measured using a Histamine ELISA (IBL, Hamburg, Germany). IL-8 was measured using a PeliKine Compact™ human IL-8 ELISA kit from Sanquin Reagents (Amsterdam, The Netherlands). For IL-8, all values were corrected for their control, which was set at 100%. Measured values for IL-8 were between 5 and 150 pg/ml.

2.8. Statistical analysis

All data (except histamine) were corrected for the control samples (no stimulation, but incubated during the same time) of the respective day of stimulation (control=100%). Statistics were performed using a non-parametric Mann–Whitney *U* test, since there were low numbers of samples. A value of $p < 0.05$ was considered significant.

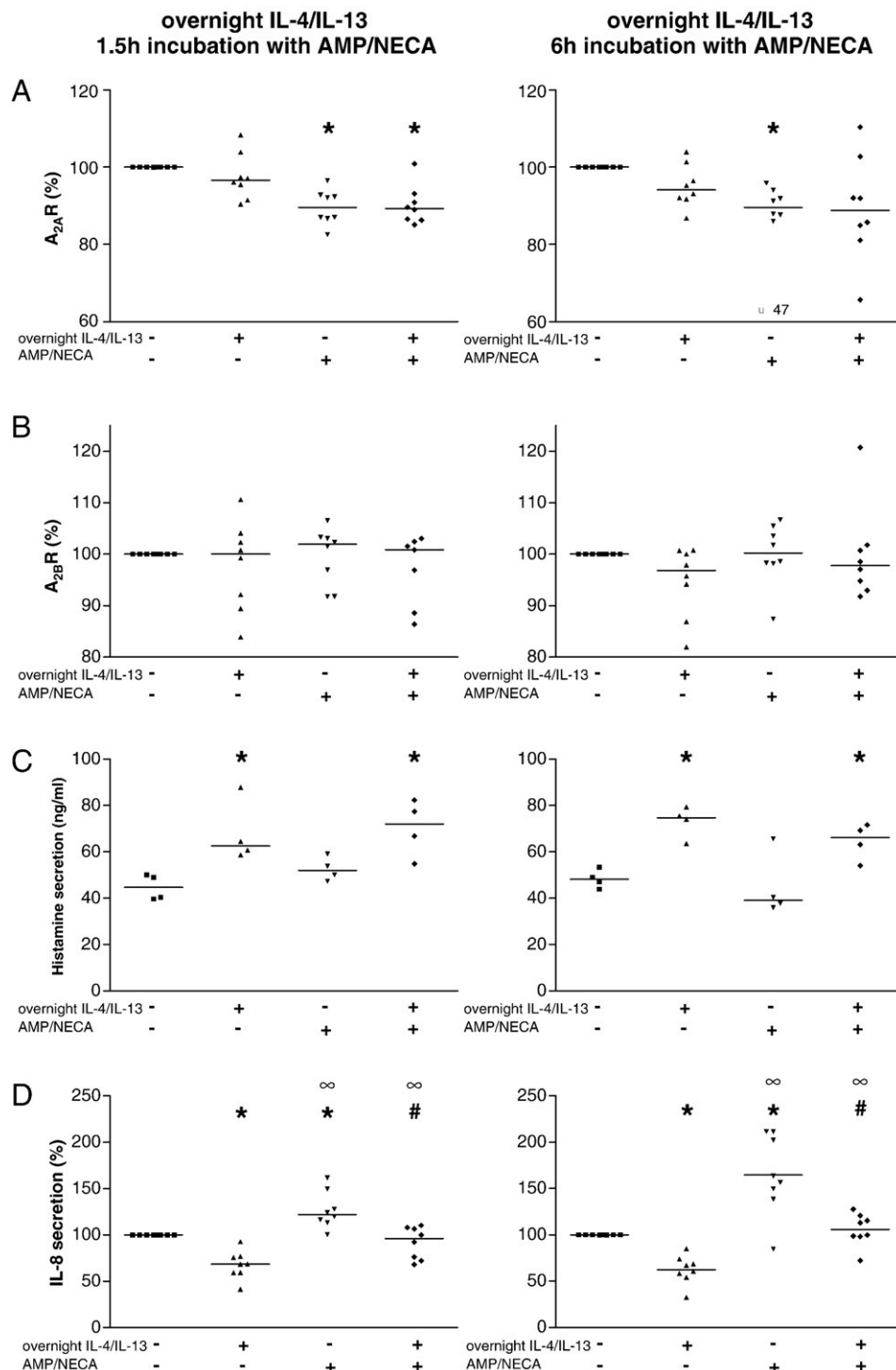


Figure 3 Adenosine A_{2A} (A) and A_{2B} (B) receptor expression, histamine release (C), and IL-8 secretion (D) after overnight incubation of HMC-1 cells with IL-4/IL-13, and subsequently AMP/NECA stimulation for 1.5 h (left panels) and 6 h (right panels). All values (except histamine) are corrected for the control which was set to 100%. Horizontal bars represent median values. * $p < 0.05$ vs. control, $\infty p < 0.05$ vs. overnight IL-4/IL-13, # $p < 0.05$ vs. AMP/NECA.

3. Results

3.1. Effects of AMP and NECA

AMP and NECA, or their combination induced no changes in the adenosine A_{2A} and A_{2B} receptor expression when compared to control (Fig. 1A and B).

There was some basal histamine release, and stimulation of HMC-1 cells with AMP/NECA for 1.5 h enhanced histamine release ($p=0.05$).

To compare our results with those described in the literature where IL-8 was described as a marker for HMC-1 activation via $A_{2B}R$ (19;21), we measured IL-8 in our supernatants. Incubation with NECA or the combination of AMP and NECA significantly enhanced IL-8 secretion by HMC-1 cells after 1.5 and 6 h incubation ($p<0.05$, Fig. 1D), no synergistic effect of AMP and NECA was observed. Incubation with AMP for 6 h increased IL-8 secretion slightly, but significantly.

3.2. Effects of IL-4 and IL-13

Stimulation with AMP and NECA-induced minimal changes in adenosine receptor expression. Therefore, HMC-1 cells were incubated with IL-4 and IL-13, cytokines that are known to be present in asthmatic lungs, to assess their effect on adenosine receptor expression and responsiveness. IL-4 and IL-13 separately had no effect on the expression of both adenosine A_2 receptors (Fig. 2A and B). In contrast, we found a modest increased adenosine $A_{2A}R$ expression after 1.5 and 6 h ($p<0.05$) and of $A_{2B}R$ expression after 6 h ($p<0.05$) when cells were stimulated with the combination of IL-4 and IL-13. $A_{2B}R$ expression was increased after 6 h of incubation with the combination of IL-4/IL-13 and AMP/NECA ($p<0.05$, Fig. 2A and B). Increased histamine release was found after incubation with IL-4 or the combination of IL-4 and IL-13 for 1.5 h and 6 h and the combination of IL-4/IL-13/AMP/NECA for 1.5 h ($p<0.05$, Fig. 2C).

In contrast to the increased IL-8 secretion after incubation with AMP and NECA, IL-8 secretion was significantly decreased after incubation with IL-4 and IL-13 alone for both 1.5 and 6 h and the combination of IL-4/IL-13 for 6 h ($p<0.05$, Fig. 2D). When cells were incubated with the combination of IL-4/IL-13/AMP/NECA, IL-8 secretion increased significantly when compared to control and IL-4/IL-13 ($p<0.05$).

3.3. Effects of overnight incubation with IL-4 and IL-13

To further enhance adenosine responsiveness with respect to AR expression and histamine release, HMC-1 cells were incubated with IL-4 and IL-13 for a longer period (18 h, overnight), and subsequently stimulated with AMP/NECA for 1.5 or 6 h. We found a downregulated $A_{2A}R$ expression after overnight IL-4/IL-13 incubation and subsequent AMP/NECA stimulation for 1.5 (Fig. 3A, $p<0.05$) with no effect on $A_{2B}R$ expression (Fig. 3B). However, the $A_{2A}R/A_{2B}R$ ratio was significantly ($p<0.05$) decreased after overnight incubation with IL-4/IL-13 and subsequent stimulation with AMP/NECA for 1.5 or 6 h, being (median (range)) 0.90 (0.85–1.07) and 0.89 (0.54–0.98) respectively. This was accompanied by an enhanced histamine release when compared to control ($p<0.05$, Fig. 3C).

Enhanced histamine release was also found after stimulation with IL-4/IL-13 alone ($p<0.05$, Fig. 3C). Overnight IL-4/IL-13 incubation

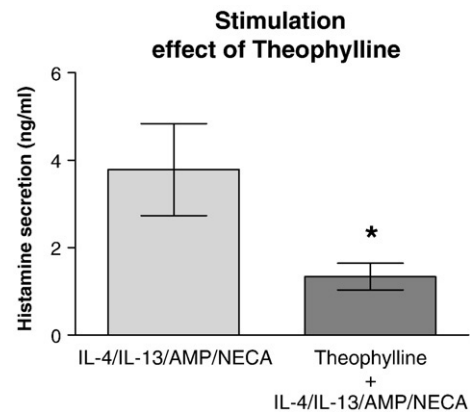


Figure 4 Histamine secretion by HMC-1 cells pre-incubated for 30 min with theophylline and subsequently stimulated for 6 h with IL-4/IL-13 and AMP/NECA. Data expressed as mean $\pm$ SEM. * $p<0.05$ vs. IL-4/IL-13/AMP/NECA.

decreased IL-8 production when compared to control ($p<0.05$, Fig. 3D), as well as AMP/NECA-induced IL-8 production ($p<0.05$, Fig. 3D).

3.4. Effects of theophylline

In order to confirm a role for the adenosine A_{2B} receptor in histamine secretion by HMC-1 cells, cells were incubated with theophylline prior to stimulation. As shown in Fig. 4, theophylline protected against IL-4/IL-13/AMP/NECA-induced histamine secretion. Theophylline alone had no effect on viability and histamine release of HMC-1 cells (data not shown).

4. Discussion

In order to shed more light on the enhanced adenosine responsiveness of asthma patients [1,22,23], we studied adenosine receptor expression and responsiveness of HMC-1 mast cells in relation to various culture conditions *in vitro*. Most interestingly, we found that increased levels of histamine were released when HMC-1 cells were cultured in the presence of cytokines that are important in the pathogenesis of asthma, i.e. IL-4 and IL-13, in combination with AMP and NECA. The highest increase in histamine release was found when cells were cultured for a longer period with IL-4 and IL-13, i.e. overnight (18 h), followed by AMP/NECA. This enhanced histamine release after overnight stimulation was accompanied by a decrease in the A_{2A}/A_{2B} receptor expression ratio on the cell, due to a decreased $A_{2A}R$ expression. Since the A_{2B} receptor is involved in NECA-induced mast cell degranulation [21] whereas the A_{2A} receptor is involved in inhibition of the release of histamine from mast cells [10,24], the ratio between the two receptors may give additional information on the sensitivity of HMC-1 cells for AMP/NECA and IL-4/IL-13 stimulation. Although no data in the literature are available on the $A_{2A}R/A_{2B}R$ ratio of mast cells in asthma, a shift in the A_{2A}/A_{2B} receptor ratio towards the $A_{2B}R$ may explain the enhanced sensitivity of asthma patients to inhaled AMP, caused by a decreased suppression of degranulation by the $A_{2A}R$.

Enhanced histamine release was also found, albeit to a smaller extent, when cells were cultured with the combination of AMP and NECA (1.5 h). Our results support earlier data,

showing that not only adenosine, but also NECA, cause an enhanced histamine release from lavage mast cells in 37 of 54 asthma patients [25]. This could be inhibited after pre-incubation of lavage mast cells with an adenosine receptor antagonist.

We showed that theophylline reduced the IL-4/IL-13/AMP/NECA-induced histamine release. It has been described that theophylline inhibits phosphodiesterases, thereby increasing intracellular cAMP levels of mast cells which results in inhibition of mediator release [26,27]. It has been suggested that degranulation of mast cells upon stimulation with AMP or NECA is mediated by A_{2B} R on HMC-1 cells. Since we show that theophylline inhibits histamine secretion upon IL-4/IL-13/AMP/NECA stimulation, it is likely that theophylline antagonizes adenosine receptors.

The effect of IL-4 or IL-13 on histamine release, adenosine responsiveness, and adenosine receptor expression of HMC-1 cells has not been described before. It is known that both cytokines can induce airway hyperresponsiveness within 6 h after administration to airways of naïve (unstimulated) mice [28]. IL-4 has been described to enhance IgE/anti-IgE antibody-induced histamine release in human cultured mast cells that were generated from umbilical cord blood mononuclear cells [29]. Recently, Ma *et al.* [30] showed in IL-4 transgenic mice that IL-4 stimulates tissue adenosine accumulation, inhibits adenosine deaminase (the enzyme responsible for adenosine breakdown to inosine) activity, and enhances the expression of the adenosine A_1 , A_{2B} , and A_3 receptors in C57BL/6 mice. These results clearly confirm the connection between IL-4 and adenosine receptor expression as found in our study, although Ma *et al.* did not investigate the expression site of the adenosine receptors. The same study also showed that IL-4 differentially regulated adenosine metabolism and receptor expression in two different mouse strains, demonstrating that genetic factors may have profound influences on effects of IL-4. Thus differences in the regulation of adenosine metabolism and receptor expression may contribute to differences in adenosine responsiveness.

In a study using IL-13 transgenic mice, IL-13 caused striking changes in adenosine receptor mRNA expression of the lung, the A_{2A} receptor expression being unaltered or decreased and A_1 , A_{2B} , and A_3 receptors being increased in their expression [31]. These results clearly suggest a relation between IL-13 and adenosine receptor expression, but as for IL-4, expression sites of the receptors were not investigated.

With respect to the effect of IL-4/IL-13 on adenosine receptor expression in this study, overnight stimulation with IL-4/IL-13 in combination with AMP/NECA for 1.5 h down-regulated A_{2A} R expression (Fig. 3), accompanied by enhanced histamine release. This finding was not seen after stimulation with AMP/NECA alone (Fig. 1), suggesting that IL-4/IL-13 interacts with the mast cell and potentiates adenosine-induced mast cell activation, by downregulation of the A_{2A} R.

We measured IL-8 next to histamine since IL-8 is produced by HMC-1 cells upon adenosine stimulation [18,19,21]. IL-8 is a member of the CXC chemokine family involved in recruitment of leukocytes to the site of inflammation [32]. Unstimulated HMC-1 cells secrete a very low amount of IL-8 but its production is rapidly induced by stimuli like e.g. adenosine [19,21]. The primary adenosine receptor involved is likely the A_{2B} receptor since agonists (e.g. NECA) with the strongest binding to the A_{2B} receptor were the best stimulators of IL-8

release, and furthermore adenosine-mediated IL-8 production can be inhibited by the competitive A_{2B} R antagonists theophylline (non-selective) and enprofylline [19,21,33]. Our data regarding IL-8 secretion upon AMP or NECA stimulation are in line with this. Interestingly, IL-8 production was also *inhibited* after culturing of HMC-1 cells in the presence of IL-4 and IL-13 or their combination (Figs. 2 and 3). This is compatible with the following observations. IL-13 inhibits IL-8 release in resting synovial fibroblasts [34] and IL-4 decreases TNF- α -induced IL-8 mRNA expression in the keratinocyte cell line HaCaT [35]. Additionally, IL-4 and IL-13 are potent inhibitors of IL-1 β induced IL-8 secretion by human intestinal epithelial cells [36]. The mechanisms underlying the decreased IL-8 release under influence of IL-4 and IL-13 are as yet unknown, but they can be various in nature since both cytokines can signal through multiple pathways.

In summary, we have described a mast cell model in which overnight (18 h) culturing of HMC-1 cells in the presence of IL-4/IL-13 and AMP/NECA results in enhanced histamine release, which is accompanied by decreased A_{2A} receptor expression and A_{2A} / A_{2B} receptor ratio. When translating this to the human situation, this mast cell model may mimic enhanced adenosine responsiveness that is present in asthma, suggesting a role for decreased A_{2A} R expression.

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