

## Research article

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# Suppression of inflammation by low-dose methotrexate is mediated by adenosine A<sub>2A</sub> receptor but not A<sub>3</sub> receptor activation in thioglycollate-induced peritonitis

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## Abstract

Prior studies demonstrate that adenosine, acting at one or more of its receptors, mediates the anti-inflammatory effects of methotrexate in animal models of both acute and chronic inflammation. Both adenosine A<sub>2A</sub> and A<sub>3</sub> receptors contribute to the anti-inflammatory effects of methotrexate treatment in the air pouch model of inflammation, and the regulation of inflammation by these two receptors differs at the cellular level. Because different factors may regulate inflammation at different sites we examined the effect of low-dose weekly methotrexate treatment (0.75 mg/kg/week) in a model of acute peritoneal inflammation in adenosine A<sub>2A</sub> receptor knockout mice and A<sub>3</sub> receptor knockout mice and their wild-type littermates. Following intraperitoneal injection of thioglycollate there was no significant difference in the number or type of leukocytes, tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-10 levels that accumulated in the thioglycollate-induced peritoneal exudates in adenosine A<sub>2A</sub> knockout mice or wild-type control mice. In contrast, there were more leukocytes, TNF- $\alpha$  and IL-10 in the exudates of the adenosine A<sub>3</sub> receptor-deficient mice. Low-dose, weekly methotrexate treatment increased the adenosine concentration

in the peritoneal exudates of all mice studied, and reduced the leukocyte accumulation in the wild-type mice and A<sub>3</sub> receptor knockout mice but not in the A<sub>2A</sub> receptor knockout mice. Methotrexate reduced exudate levels of TNF- $\alpha$  in the wild-type mice and A<sub>3</sub> receptor knockout mice but not the A<sub>2A</sub> receptor knockout mice. More strikingly, IL-10, a critical regulator of peritoneal inflammation, was increased in the methotrexate-treated wild-type mice and A<sub>3</sub> knockout mice but decreased in the A<sub>2A</sub> knockout mice. Dexamethasone, an agent that suppresses inflammation by a different mechanism, was similarly effective in wild-type mice, A<sub>2A</sub> mice and A<sub>3</sub> knockout mice. These findings provide further evidence that adenosine is a potent regulator of inflammation that mediates the anti-inflammatory effects of methotrexate. Moreover, these data provide strong evidence that the anti-inflammatory effects of methotrexate and adenosine are mediated by different receptors in different inflammatory loci, an observation that may explain why inflammatory diseases of some organs but not of other organs respond to methotrexate therapy.

## Introduction

Low-dose weekly methotrexate has become the mainstay treatment of rheumatoid arthritis and psoriasis, and it is the gold standard by which other systemic medications are measured in both disorders [1,2]. Methotrexate has been used to treat other inflammatory diseases including ankylosing spondylitis, multiple sclerosis and inflammatory bowel disease, but its efficacy in the therapy of these conditions is far less impressive [3-7].

An increasing body of evidence indicates that adenosine mediates, at least in part, the anti-inflammatory effects of methotrexate [8-13]. All known adenosine cell surface receptors (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>) contribute to the modulation of inflammation, as demonstrated by many *in vitro* and *in vivo* pharmacologic studies (reviewed in [14,15]). We have previously demonstrated pharmacologically, using nonselective antagonists, that the anti-inflammatory effect of methotrexate is mediated by more than one subtype of adenosine receptor in the adjuvant arthritis model in the rat [16], and, using mice ren-

ELISA = enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; IL = interleukin; PBS, phosphate-buffered saline; PCR = polymerase chain reaction; TNF- $\alpha$  = tumor necrosis factor alpha.

dered deficient in A<sub>2A</sub> or A<sub>3</sub> adenosine receptors, we found that both receptor subtypes are critical for the anti-inflammatory effects of methotrexate in the murine air pouch model of inflammation [17]. Since inflammation at different loci may be regulated by different cellular mechanisms, we determined whether the A<sub>2A</sub> and A<sub>3</sub> receptors played similar roles in regulating inflammation in the peritoneum.

We examined the pharmacologic mechanism by which methotrexate diminishes inflammation in the thioglycollate-induced peritoneal inflammation model of acute inflammation in the mouse. We report here that, similar to the air pouch, methotrexate treatment increases peritoneal exudate adenosine concentrations in wild-type mice, A<sub>2A</sub> receptor knockout mice and A<sub>3</sub> receptor knockout mice but, in contrast to the air pouch model, diminishes leukocyte accumulation only in the peritoneal exudates of A<sub>3</sub> receptor knockout and wild-type mice, not of A<sub>2A</sub> knockout mice. Similarly, methotrexate decreased exudate tumor necrosis factor alpha (TNF- $\alpha$ ) levels and increased IL-10 levels in wild-type mice and A<sub>3</sub> knockout mice, but only marginally decreased TNF- $\alpha$  levels and significantly decreased IL-10 levels in A<sub>2A</sub> knockout mice.

## Materials and methods

### Materials

Thioglycollate medium (FTG) was obtained from Sigma Chemical Co. (St Louis, MO, USA). Methotrexate was purchased from Immunex (San Juan, PR, USA). All other materials were the highest quality that could be obtained.

### Animals

Mice with a targeted disruption of the gene for the adenosine A<sub>2A</sub> and A<sub>3</sub> receptor have been described in detail elsewhere [18,19]. The mice used in these experiments were derived from four original heterozygous breeding pairs for each mouse strain. Mice described as wild type were specific for the related receptor knockout mice, since their background was different. Confirmation of mouse genotype was performed by PCR as previously described [17]. Mice were housed in the New York University animal facility, fed regular mouse chow and given access to drinking water *ad libitum*. All procedures described in the following were reviewed and approved by the Institutional Animal Care and Use Committee of New York University Medical Center and were carried out under the supervision of the facility veterinary staff.

### Peritoneal inflammation

Animals were given weekly intraperitoneal injections of either methotrexate (0.75 mg/kg, freshly reconstituted lyophilized powder) or vehicle (0.9% saline) for 4 weeks and the experiments were carried out within 3 days of the final dose of methotrexate. Dexamethasone (1.5 mg/kg) was administered by intraperitoneal injection 1 hour prior to induction of inflammation in the peritoneum. Thioglycollate peritonitis was induced by intraperitoneal injection of 0.5 ml sterile solution of thiogly-

collate medium (10% w/v in PBS) [20]. After 4 hours the animals were sacrificed by CO<sub>2</sub> narcosis and their peritoneal cavities were lavaged with 3 ml cold PBS. The peritoneal area was massaged before withdrawing the lavage fluid. Exudates were maintained at 4°C until aliquots were diluted 1:1 with methylene blue (0.01% w/v in PBS) and cells were counted in a standard hemocytometer chamber. The concentration of adenosine and TNF- $\alpha$  in inflammatory exudates was quantified by HPLC and ELISA, respectively [17]. The IL-10 concentration in cell-free inflammatory exudates was quantified by ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

### Statistical analysis

All statistical analyses were performed by SigmaStat software (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed by one-way analysis of variance.

## Results

Since previous studies carried out in our laboratory showed that adenosine receptors play a pivotal role in the formation of the granulation tissue lining the air pouch [21], in a manner that might alter the inflammatory response, we sought to further evaluate the role of adenosine receptors in methotrexate-mediated suppression of inflammation in tissue that had not previously undergone injury or disruption. We therefore determined whether methotrexate inhibits acute leukocyte accumulation in thioglycollate-induced peritoneal inflammation in wild-type mice, adenosine A<sub>2A</sub> receptor knockout mice and adenosine A<sub>3</sub> receptor knockout mice. Similar numbers of leukocytes accumulated in peritoneal inflammatory exudates of A<sub>2A</sub> knockout mice and their corresponding wild-type controls (Table 1). In contrast, there was a significant increase (20%) in the number of leukocytes that accumulated in peritoneal exudates of A<sub>3</sub> knockout mice as compared with the wild-type controls (Table 1).

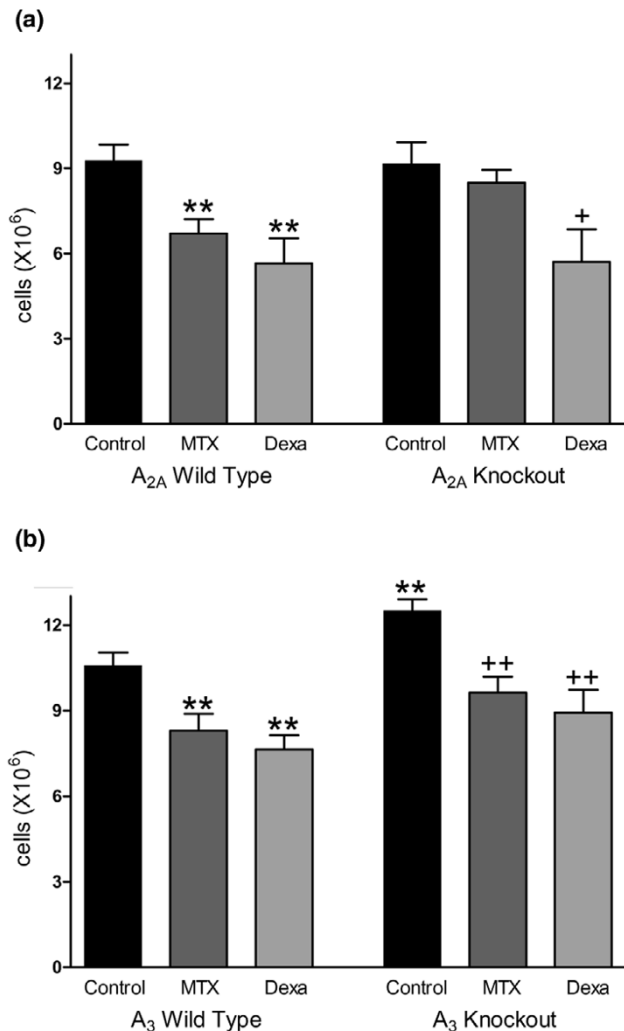
Treatment with methotrexate increased the exudate adenosine concentration in wild-type mice, A<sub>2A</sub> knockout mice and A<sub>3</sub>

**Table 1**

### Leukocyte accumulation in inflammatory exudates

| Mouse group               | Peritoneal exudate ( $\times 10^6$ cells $\pm$ SEM) |
|---------------------------|---|
| A <sub>2A</sub> wild type | 9.3 $\pm$ 0.6 (n = 14)                              |
| A <sub>2A</sub> knockout  | 9.2 $\pm$ 0.8 (n = 14)                              |
| A <sub>3</sub> wild type  | 10.6 $\pm$ 0.5 (n = 19)                             |
| A <sub>3</sub> knockout   | 12.5 $\pm$ 0.4* (n = 23)                            |

Inflammatory exudates were induced in the peritoneum of knockout and wild-type mice, as described. After 4 hours the exudates were collected and the leukocytes quantitated. The wild-type control mice were derived from the same heterozygous breeding pairs and were matched for age and sex. There was no difference in the number of leukocytes accumulating in the exudates of male vs female mice in either the knockout mice or wild-type mice. \* $P < 0.005$  vs A<sub>3</sub> wild-type mice, Student's *t* test.

**Figure 1**

Effect of methotrexate and dexamethasone treatment on leukocyte accumulation in peritoneal exudates of mice. **(a)** A<sub>2A</sub> wild-type mice and A<sub>2A</sub> receptor knockout mice or **(b)** A<sub>3</sub> wild-type mice and A<sub>3</sub> receptor knockout mice either were treated with weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation or were treated with a single intraperitoneal injection of dexamethasone (1.5 mg/kg) or saline 1 hour before induction of inflammation and subsequent collection of inflammatory exudates, as described. Results are presented as the mean ( $\pm$  SEM) million cells per exudate. \*\* $P < 0.001$  vs wild-type control mice, ++ $P < 0.001$  vs knockout control mice, + $P < 0.05$  vs knockout control mice, all one-way analysis of variance (Bonferroni  $t$  test).

knockout mice (Table 2) and reduced the leukocyte accumulation in A<sub>2A</sub> wild-type mice by  $30 \pm 5\%$  ( $P < 0.01$  vs control,  $n = 7$ ; Figure 1a), but reduced the leukocyte accumulation in the A<sub>2A</sub> knockout mice by only  $7 \pm 5\%$  ( $P =$  not significant vs wild-type control,  $n = 6$ ; Figure 1a). In contrast to the A<sub>2A</sub> knockout mice, methotrexate was no less effective as an anti-inflammatory agent in A<sub>3</sub> receptor knockout mice ( $23 \pm 5\%$  inhibition,  $P < 0.001$  vs A<sub>3</sub> knockout control,  $n = 12$ ; Figure

1b) than in A<sub>3</sub> wild-type mice ( $22 \pm 5\%$  inhibition,  $P < 0.001$  vs A<sub>3</sub> wild-type control,  $n = 10$ ; Figure 1b).

To determine whether the diminished anti-inflammatory effect of methotrexate in the A<sub>2A</sub> knockout mice was specific, we tested the effect of the potent steroidal anti-inflammatory agent dexamethasone in this model. Dexamethasone diminished leukocyte accumulation similarly in A<sub>2A</sub> wild-type mice, A<sub>2A</sub> knockout mice, A<sub>3</sub> wild-type mice and A<sub>3</sub> knockout mice ( $39 \pm 9\%$ ,  $38 \pm 13\%$ ,  $35 \pm 4\%$  and  $36 \pm 4\%$  inhibition,  $P < 0.005$ ,  $P < 0.05$ ,  $P < 0.001$  and  $P < 0.001$  vs control,  $n = 4$ ,  $n = 3$ ,  $n = 9$  and  $n = 9$ , respectively; Figure 1). Under the conditions studied there was no difference in the type of white cells that accumulated in the peritoneal cavities of either treated or untreated wild-type mice or knockout mice (>90% polymorphonuclear leukocytes).

In general, TNF- $\alpha$  accumulation in peritoneal exudates was much lower than previously reported in other models of inflammation, including carrageenan-induced inflammation in the air pouch and zymosan-induced peritoneal inflammation [17,22]. Similar to leukocyte accumulation, we found comparable levels of the proinflammatory cytokine TNF- $\alpha$  in peritoneal exudates of wild-type mice and A<sub>2A</sub> knockout mice, but significantly increased accumulation of TNF- $\alpha$  in peritoneal exudates of A<sub>3</sub> knockout mice (Table 3). Methotrexate nevertheless inhibited TNF- $\alpha$  accumulation in peritoneal exudates of wild-type mice and A<sub>3</sub> knockout mice more markedly than leukocyte accumulation (by 67% and 59%, respectively), and had a modest effect on TNF- $\alpha$  accumulation in peritoneal exudates of A<sub>2A</sub> knockout mice (Table 3). These findings are consistent with the prior observation that both A<sub>2A</sub> and A<sub>3</sub> receptors modulate TNF- $\alpha$  production [23].

The cytokine IL-10, released by resident peritoneal macrophages, plays a regulatory anti-inflammatory role in the recruitment of leukocytes in murine models of peritoneal inflammation [22,24]. Since adenosine receptor activation modulates the release of IL-10 by different inflammatory cells [25-27] and methotrexate-treated rheumatoid arthritis patients have shown increased serum levels of this cytokine [28,29], we determined whether constitutively or methotrexate-modified IL-10 accumulation in the inflammatory exudate was altered in adenosine receptor-deficient mice. We found that, similar to the leukocyte infiltration and the TNF- $\alpha$  concentration, A<sub>3</sub> knockout mice had significantly higher IL-10 levels in their peritoneal inflammatory exudates when compared with wild-type mice and A<sub>2A</sub> knockout mice (Table 4). As expected, treatment with methotrexate stimulated IL-10 accumulation in the exudate by 56% in wild-type mice, but significantly decreased IL-10 levels in exudates of A<sub>2A</sub>-deficient mice. Although methotrexate increased IL-10 levels in the exudates of methotrexate-treated A<sub>3</sub> knockout mice, this increase did not achieve statistical significance. Due to the high variability in the IL-10 levels we found in our experiments, it would

**Table 2****Adenosine concentration in peritoneal exudates**

|                                | Wild-type mice (nM ± SEM) | A <sub>2A</sub> knockout mice (nM ± SEM) | A <sub>3</sub> knockout mice (nM ± SEM) |
|--------------------------------|---------------------------|--|---|
| Control                        | 118 ± 6 (n = 19)          | 110 ± 6 (n = 14)                         | 133 ± 6 (n = 12)                        |
| Methotrexate (0.75 mg/kg/week) | 178 ± 12* (n = 15)        | 162 ± 7** (n = 7)                        | 214 ± 10† (n = 9)                       |

Wild-type mice, A<sub>2A</sub> receptor knockout mice or A<sub>3</sub> receptor knockout mice were treated with either weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation. Inflammatory exudates were induced in the peritoneum of mice, as described. After 4 hours the exudates were collected and the adenosine levels quantitated. Wild-type data are a combination from both mouse strains. \**P* < 0.0001 vs wild-type control mice, Student's *t* test; \*\**P* < 0.0001 vs A<sub>2A</sub> knockout control mice, Student's *t* test; †*P* < 0.0001 vs A<sub>3</sub> knockout control mice, Student's *t* test.

require between 30 and 60 mice per group to achieve statistical significance.

These results provide evidence that the anti-inflammatory effects of methotrexate (and adenosine) are mediated by different receptors in different loci. Specifically, in contrast to our previously published observation that both A<sub>2A</sub> and A<sub>3</sub> receptors are required for the anti-inflammatory effects in the air pouch model of inflammation, only the A<sub>2A</sub> receptor is required to suppress inflammation in the peritoneal space.

## Discussion

The purine nucleoside adenosine is a ubiquitous autacoid present in all tissues and body fluids. Under basal conditions, the extracellular adenosine concentration is rather constant (30–300 nM), but its concentration can increase dramatically to 10 μM or even higher, as a result of ATP catabolism, when there is an imbalance between energy use and energy supply, such as in oxygen depletion, or when there is cell necrosis as a consequence of mechanical or inflammatory injury. Adenosine acts via four distinct adenosine receptor subtypes – the adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors – that are all members of the large family of seven-transmembrane spanning, heterotrimeric G protein-associated receptors, coupling to classical second messenger pathways such as modulation of cAMP production or the phospholipase C pathway. In addition, they couple to mitogen-activated protein kinases, which could give them a role in cell growth, survival, death and differentiation (reviewed in [30]).

Adenosine is a potent endogenous anti-inflammatory agent, and all four adenosine receptor subtypes participate in this effect (reviewed in [14]). All cell subtypes involved in the inflammatory process differentially express functional adenosine receptors. It is well documented that microvascular endothelial cells, major players conducting the movement of leukocytes between tissue compartments, express adenosine A<sub>2A</sub> and A<sub>2B</sub> receptors [31,32]. Pharmacological and molecular approaches have shown that neutrophils, monocytes and macrophages express all four adenosine receptor subtypes. Although adenosine A<sub>1</sub> receptor activation has been associated with proinflammatory properties in inflammatory cell types [33–35], the anti-inflammatory effect of selective A<sub>1</sub> agonists

acting in the central nervous system has been demonstrated *in vivo* [36–38]. Adenosine A<sub>2A</sub> receptor activation inhibits neutrophil and monocyte oxidative burst, degranulation and release of cytokines and chemokines [39–41]. Activation of A<sub>2B</sub> receptors selectively inhibits collagenase mRNA accumulation in synovial fibroblasts and mediates neutrophil-stimulated intestinal epithelial leakiness [42,43]. Adenosine A<sub>3</sub> receptors have also been described as anti-inflammatory in human blood leukocytes and in murine models of inflammation [19,44–46].

The results of these reported studies confirm the anti-inflammatory effects of adenosine acting at A<sub>3</sub> receptors because animals deficient in this receptor show an exacerbated response to the inflammatory insult. Moreover, we found that more polymorphonuclear leukocytes accumulate in the peritoneal exudates of A<sub>3</sub> knockout mice in comparison with their wild-type littermates, consistent with the hypothesis that this receptor plays a greater role as an endogenous regulator of inflammation. Our data are in agreement with prior reports showing that adenosine A<sub>3</sub> receptor agonists suppress the expression and production of macrophage inflammatory protein 1α, a chemokine that enhances neutrophil recruitment into inflammatory sites [45], and suppress the production of TNF-α by lipopolysaccharide-stimulated macrophages [19]. Adenosine A<sub>3</sub> receptor agonists thus ameliorate joint inflammation in several murine models of arthritis [45,46].

Monocytes and macrophages synthesize and release into their environment a variety of cytokines and other proteins that play a central role in the development of acute and chronic inflammation. It has been firmly established that adenosine modulates the production of inflammatory cytokines, including TNF-α, IL-10, and IL-12 [23,25–27,47]. In addition to the regulatory effect of adenosine in cytokine secretion, we have further established that Th1 proinflammatory cytokine IL-1 and TNF-α treatment increases message and protein expression of A<sub>2A</sub> and A<sub>2B</sub> receptors by both microvascular endothelial cells and THP-1 monocytoïd cells. IFN-γ treatment also increased the expression of A<sub>2B</sub> receptors, but decreased the expression of A<sub>2A</sub> receptors [25,32,48]. It is therefore probably at inflamed sites, where proinflammatory cytokines such as IL-1 and TNF-α are abundantly secreted, mostly by monocytes/macro-

**Table 3****Tumor necrosis factor alpha concentration in peritoneal exudates**

|                                | Wild-type mice (pg/ml $\pm$ SEM) | A <sub>2A</sub> knockout mice (pg/ml $\pm$ SEM) | A <sub>3</sub> knockout mice (pg/ml $\pm$ SEM) |
|--------------------------------|----------------------------------|---|--|
| Control                        | 42 $\pm$ 7 (n = 14)              | 36 $\pm$ 8 (n = 10)                             | 75 $\pm$ 18* (n = 6)                           |
| Methotrexate (0.75 mg/kg/week) | 14 $\pm$ 4** (n = 15)            | 25 $\pm$ 7 (n = 9)                              | 31 $\pm$ 11 <sup>†</sup> (n = 8)               |

Wild-type mice, A<sub>2A</sub> receptor knockout mice or A<sub>3</sub> receptor knockout mice were treated with either weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation. Inflammatory exudates were induced in the peritoneum of mice, as described. After 4 hours the exudates were collected, centrifuged at 100  $\times$  g and frozen. Tumor necrosis factor alpha levels were later quantitated by ELISA. Wild-type data are a combination from both mouse strains. \*\* $P < 0.001$  vs wild-type control mice, Student's *t* test; \* $P < 0.05$  vs wild-type control mice, Student's *t* test; <sup>†</sup> $P < 0.05$  vs A<sub>3</sub> knockout control mice, Student's *t* test.

phages, that the subsequent upregulation of A<sub>2A</sub> and A<sub>2B</sub> receptors on endothelial cells and other inflammatory cells along with endogenous adenosine release constitutes a feedback loop to suppress further inflammation. The demonstration that adenosine receptors expressed in microvascular endothelial cells are modified during inflammation suggests an important role for these receptors in the increased angiogenesis and vascular permeability that characterize both acute and chronic inflammatory responses. Moreover, in previous studies, activation of both A<sub>2A</sub> and A<sub>2B</sub> receptors on either endothelial cells or macrophages has been reported to enhance the expression of vascular endothelial growth factor and to promote angiogenesis [21,49-51].

Methotrexate is an effective disease-modifying drug widely used in low doses at weekly intervals for the control of rheumatoid arthritis and psoriasis with a relatively safe profile compared with other therapies [1,2]. Since folate administration prevents many of the toxicities of methotrexate without affecting the therapeutic effects [52], there is little support for the hypothesis that inhibition of folate-dependent pathways (for example, cellular proliferation) is responsible for the therapeutic effects of the agent. Following administration, methotrexate is taken up by cells and undergoes polyglutamation, resulting in the intracellular accumulation of the long-lived polyglutamates of methotrexate. These metabolites, in addition to inhibiting folate metabolism, directly inhibit 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, resulting in an intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide, which is an intermediate metabolite in the *de novo* pathway of purine synthesis, and has been associated with increases in extracellular adenosine [9,13,53].

There is now increasing evidence that accumulation of adenosine at sites of inflammation plays a pivotal role in the anti-inflammatory effect of methotrexate. *In vitro* studies showed that methotrexate produces adenosine release by human fibroblasts and endothelial cells [53], and *in vivo* studies showed that methotrexate is ineffective in the presence of antagonists of adenosine or adenosine deaminase (the enzyme responsible for the deamination of adenosine to inosine) in animal models of acute and chronic inflammation [8]. Moreover, adenosine receptor antagonists and deletion of adenosine receptors eliminates the anti-inflammatory response to methotrexate in animal models of acute and chronic inflammation and patients with rheumatoid arthritis [13,16,54].

Although the contribution of adenosine to the mechanism of action of methotrexate is well accepted, it is still unclear which adenosine receptors participate in the effect of methotrexate. Results of early studies, using pharmacological tools, suggested that the adenosine A<sub>2A</sub> receptor was the main receptor subtype involved in suppressing inflammation [8]. In the model of adjuvant arthritis in rats, however, we found that only nonselective adenosine receptor antagonists could block the protective effect of methotrexate whereas selective antagonists of individual adenosine receptors did not alter the response to methotrexate [16], consistent with involvement of multiple adenosine receptors. Using knockout animals we observed that both A<sub>2A</sub> and A<sub>3</sub> adenosine receptors are involved in methotrexate-mediated suppression of air pouch inflammation [17] but, as reported here, only A<sub>2A</sub> receptors are involved in methotrexate-mediated suppression of peritoneal inflammation. Methotrexate exerted similar anti-inflammatory effects in wild-

**Table 4****IL-10 concentration in peritoneal exudates**

|                                | Wild-type mice (pg/ml $\pm$ SEM) | A <sub>2A</sub> knockout mice (pg/ml $\pm$ SEM) | A <sub>3</sub> knockout mice (pg/ml $\pm$ SEM) |
|--------------------------------|----------------------------------|---|--|
| Control                        | 62 $\pm$ 7 (n = 24)              | 73 $\pm$ 9 (n = 12)                             | 115 $\pm$ 14** (n = 15)                        |
| Methotrexate (0.75 mg/kg/week) | 97 $\pm$ 18* (n = 12)            | 41 $\pm$ 6 <sup>†</sup> (n = 7)                 | 150 $\pm$ 31 (n = 7)                           |

Wild-type mice, A<sub>2A</sub> receptor knockout mice or A<sub>3</sub> receptor knockout mice were treated with either weekly injections of methotrexate (0.75 mg/kg) or saline control for 4 weeks prior to induction of inflammation. Inflammatory exudates were induced in the peritoneum of mice, as described. After 4 hours the exudates were collected, centrifuged at 100  $\times$  g and frozen. IL-10 levels were later quantitated by ELISA. Wild-type data are a combination from both mouse strains. \*\* $P < 0.001$  vs wild-type control mice, Student's *t* test; \* $P < 0.05$  vs wild-type control mice, Student's *t* test; <sup>†</sup> $P < 0.05$  vs A<sub>2A</sub> knockout control mice, Student's *t* test.

type mice and A<sub>3</sub> knockout mice, but failed to inhibit leukocyte and TNF- $\alpha$  accumulation in A<sub>2A</sub> knockout mice. Moreover, methotrexate treatment augmented the accumulation of IL-10, a known anti-inflammatory cytokine, in wild-type mice and A<sub>3</sub> knockout mice, but actually decreased IL-10 levels in A<sub>2A</sub> knockout mice. We do not have a clear explanation for this other than to note it is probable that in the MTX-treated A<sub>2A</sub> knockout mice there is an imbalance in A<sub>1</sub> adenosine receptor function in the absence of A<sub>2A</sub>, consistent with the previous observation of Hasko and colleagues that an A<sub>1</sub> adenosine receptor agonist reduces IL-10 release by lipopolysaccharide-stimulated RAW macrophages [27]. IL-10 is therefore, as previously reported, a critical regulator of peritoneal inflammation that is regulated by A<sub>2A</sub> adenosine receptors but not by A<sub>3</sub> adenosine receptors [24,25].

We infer from these results and previous reports that the involvement of different adenosine receptor subtypes depends upon the site of and stimulus for inflammation. We therefore conclude it is probable that the requirement for activation of multiple adenosine receptor subtypes in the pharmacologic control of chronic inflammation results from the involvement of different types of inflammatory cells and disease-specific differences in the inflammatory environment.

## Conclusion

The studies reported here provide strong evidence that adenosine mediates the anti-inflammatory effects of methotrexate at doses relevant to those used to treat inflammatory arthritis. These results indicate that agents which interact with adenosine A<sub>2A</sub> receptors directly or promote adenosine release at inflamed sites may be useful for the treatment of inflammatory conditions, whereas occupancy of other adenosine receptors may be involved in suppression of inflammation in a site-specific fashion.

## Competing interests

MCM and AD declare that they have no competing interests. BNC declares the following competing interests: consultant – King Pharmaceuticals, Tap Pharmaceuticals, Can-Fite Pharmaceuticals, Bristol-Myers Squibb, Regeneron, Centocor; grant support – NIH, King Pharmaceuticals; honoraria – Merck, Amgen; intellectual property – adenosine A<sub>2A</sub> receptors for wound healing, adenosine A<sub>2A</sub> receptor antagonists for fibrosis (both licensed to King Pharmaceuticals).

## Authors' contributions

MCM designed and coordinated the study, carried out the animal experimental procedures, performed the statistical analysis and drafted the manuscript. AD carried out the adenosine HPLC determinations and the immunoassays. BNC conceived of the study, participated in its design and corrected the manuscript. All authors read and approved the final manuscript.

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