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Analysis of A2a receptor-deficient mice reveals no significant compensatory increases in the expression of A2b, A1, and A3 adenosine receptors in lymphoid organs

Dmitriy E. Lukashev, Patrick T. Smith, Charles C. Caldwell, Akio Ohta, Sergey G. Apasov, Michail V. Sitkovsky*

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 10 Center Drive, Room 11N-256, Bethesda, MD 20892, USA

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Abstract

Although recent genetic and pharmacologic *in vivo* studies of acute inflammation models in mice demonstrated that the cyclic AMP-elevating A2a receptor plays a non-redundant role in protection from excessive acute inflammatory tissue damage and in the down-regulation of proinflammatory cytokine production, it remained to be established whether genetic deficiency of the A2a receptor is accompanied by a compensatory up-regulation of the cAMP-elevating A2b receptor and/or other adenosine receptors. Here, we show that most of the cAMP response to adenosine is abolished in lymphoid tissues of A2a receptor-deficient mice, although some response remains in splenocytes. No significant changes were observed in A2b, A1, and A3 mRNA levels in the thymus or lymph nodes of A2a receptor-deficient mice, but small increases in mRNA expression of these receptors were detected in the spleen. These data suggest that regulation of the expression of A2b, A1, and A3 receptors is not affected significantly by the absence of A2a receptors and may provide further explanation of earlier *in vivo* observations of increased tissue damage and of longer persistence of proinflammatory cytokines in animals with inactivated A2a receptors.

Keywords: Adenosine; Adenosine receptors; Inflammation; Cyclic AMP

1. Introduction

Inflammation is a defensive response of organisms against various pathogens; such processes are crucial for survival. However, the destruction of pathogens may also involve collateral tissue damage due to actions of proinflammatory molecules [1,2]. Extended or excessive inflammation may lead to septic shock or autoimmune diseases and is implicated in the pathogenesis of cardiovascular diseases and some cancers [3–7]. Powerful anti-inflammatory mechanisms regulate inflammation by controlling the balance between pro- and anti-inflammatory molecules [8,9]. It was shown recently that extracellular Ado-mediated signaling is an important anti-inflammatory mechanism involving anti-inflammatory cytokines, prostaglandins [10,11], lipoxins [8], and glucocorticoids [12].

Intensive studies of the potential role of extracellular Ado in inflammation were facilitated by identification and cDNA cloning of four types of Ado receptors. A1 and A3 Ado receptors are G_i protein coupled, while A2a and A2b are G_s protein-coupled receptors that can activate adenylate cyclase and cause accumulation of intracellular cAMP [13]. Accumulation of Ado during ischemia and inflammation can protect normal tissues from injury due to immunosuppressive signaling through Ado receptors [14–18]. Similar protection from excessive inflammatory tissue injury by A2a receptor signaling was demonstrated *in vivo* using A2a receptor-deficient (*Adora2a*$^{-/-}$) mice [19]. It was shown that sub-threshold doses of inflammatory stimulus, which caused minimal damage in normal

* Corresponding author. Tel.: +1-301-496-5495; fax: +1-301-480-7352.
E-mail address: msitkovsky@niaid.nih.gov (M.V. Sitkovsky).
Abbreviations: Ado, adenosine; *Adora2a*$^{-/-}$, A2a receptor deficiency; cAMP, cyclic AMP; and RT–PCR, reverse transcription–polymerase chain reaction.

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mice, induced prolonged high levels of proinflammatory cytokines that led to extensive tissue damage and death of *Adora2a*$^{-/-}$ mice or normal mice treated with antagonists of the A2a receptor [19]. The role of A2a receptors seemed to be non-redundant. It is now believed that excessive inflammatory tissue damage leads to accumulation of extracellular Ado, activation of G_s-coupled A2a receptors on the surface of immune cells, and increases in immunosuppressive cAMP. This, in turn, leads to inhibition of proinflammatory cytokine secretion as well as to attenuation of inflammation. Both the A2a and A2b receptors are capable of elevating cAMP levels and thereby modulating the immune response [20]; it was therefore somewhat surprising that, in studies of acute liver inflammation, sepsis, and infected wound models, the A2b receptor was incapable of compensating for A2a receptor absence in protection from tissue damage [19]. It is not clear whether lack of compensation is due to the insufficient presence of A2b receptors in effector immune cells or to their inability to up-regulate expression sufficiently to compensate for the absence of A2a receptors. We also investigated the levels of expression of A1 and A3 receptors in *Adora2a*$^{-/-}$ mice because these receptors were also shown to have anti-inflammatory capability [20].

We designed experiments to clarify whether there are changes in expression of mRNA by cAMP-elevating A2b receptors and of G_i-coupled A1 and A3 receptors in immune cells of *Adora2a*$^{-/-}$ mice. In addition, we compared Ado-induced cAMP accumulation in immune cells from wild-type and *Adora2a*$^{-/-}$ mice. We conclude that the non-redundancy of A2a receptors in protection from excessive inflammatory tissue damage could be at least partially due to the inability of immune cells of *Adora2a*$^{-/-}$ mice to significantly up-regulate other immunosuppressive Ado receptors to compensate for their lack of A2a receptors.

2. Materials and methods

2.1. Mice

Two-month-old male C57BL/6 *Adora2a*$^{-/-}$ mice [21] and age-matched wild-type mice were maintained in pathogen-free NIH animal facilities. Three animals were used in each experiment. Mice were treated and killed according to Federal rules, regulations, and policies pertaining to the care and use of animals in medical research.

2.2. Cell subset purification

Macrophage T cell and B cell subsets from the spleen were purified using immunomagnetic separation on the AutoMACS (Miltenyi Biotec). Single cell splenocyte preparations were set upon a 6-well plate and incubated at 37° overnight. Non-adherent cells were incubated with magnetic beads conjugated with anti-CD90 mAb, separated as recommended by the manufacturer, with the positively selected cells going into the T cell subset and the negatively selected cells going into the B cell subset. Adherent cells were incubated with magnetic beads conjugated with anti-CD11b mAb and separated as recommended by the manufacturer, with the positively selected cells going into the macrophage subset. The isolated cell subsets consisted of at least 83% T cells, 80% B cells, and 75% macrophages, as judged by anti-CD4 and anti-CD8, anti-CD19, and anti-CD11b mAb staining, respectively, and analyzed by flow cytometry.

2.3. RNA extraction

Special care was taken to avoid contamination and/or degradation of RNA during separation of organs and removing blood from such organs as heart and liver. Organs (100 mg wet weight) were homogenized in 1 mL of RNA STAT-60 (Tel-Test); RNA was extracted according to the instructions of the manufacturer, followed by treatment with DNase I (Invitrogen).

2.4. Reverse transcription

First strand cDNA was synthesized on 1 μg of total RNA using the SuperSCRIPT preamplification system (Life Technologies) with random hexaprimers according to the instructions of the manufacturer. After RNase H treatment, water was added to a final volume of 100 μL.

2.5. Real-time PCR

PCR was performed in triplicates on an ABI PRISM 7700 Detection System using SYBR Green PCR Master Mix (Applied Biosystems) according to the instructions of the manufacturer. The reaction volume (25 μL) included 12.5 μL of SYBR Green PCR Master Mix, 5 μL of the diluted cDNA, and 0.2 μM of each primer. Primers for:

A1—ACAAAAACCAGTGGTGGAGTGA and TCTGTCCCCTCCCCTTGTC,
A2a—TGAAGGCGAAGGGCATCA and GGGTCAGGCCGATGGC,
A2b—ACGTGGCCGTGGGACTC and GCAGAAGCCCAAGCTGATG,
A3—GAGACCTGCATCCTCCAGGTT and GGCCTGTTACAGGACCATCAA,
β-actin—TTCAACACCCCAGCCATGTA and TGTGGTACGACCAGAGGCATAC,
L32—AGCAACAAGAAAACCAAGCACAT and TTGACATTGTGGACCAGGAACT,
GAPDH—TCAACGGGAAGCCCATCA and CGGCCTCACCCCATTTG

were synthesized by Invitrogen. Primers for 18S rRNA (Ribosomal RNA Control Reagents kit) were purchased from Applied Biosystems.

2.6. Determination of the amounts of cDNA of Ado receptors

The amounts of target cDNA were determined using the comparative C_T method. Preceding validation experiments have shown that efficiencies of target and reference are approximately equal. The ΔC_T value was determined in each experiment by subtracting the average 18S C_T value from the average C_T for Ado receptors. The standard deviation was calculated using the formula $s = \sqrt{(s_1^2 + s_2^2)}$. To set the relative unit to 1, $\Delta\Delta C_T$ was calculated by subtraction of the ΔC_T calibrator value (the largest ΔC_T value in a group). The amount of the target was calculated as the average value from $2^{-\Delta\Delta C_T+s}$ and $2^{-\Delta\Delta C_T-s}$.

2.7. Measurements of cAMP

Thymus, spleen, and lymph node cells from wild-type and *Adora2a*$^{-/-}$ mice were harvested, washed twice with complete RPMI medium containing 5% fetal bovine serum (v/v), and resuspended at 4×10^6 cells/mL in RPMI medium. The splenocyte suspension was treated with ACK

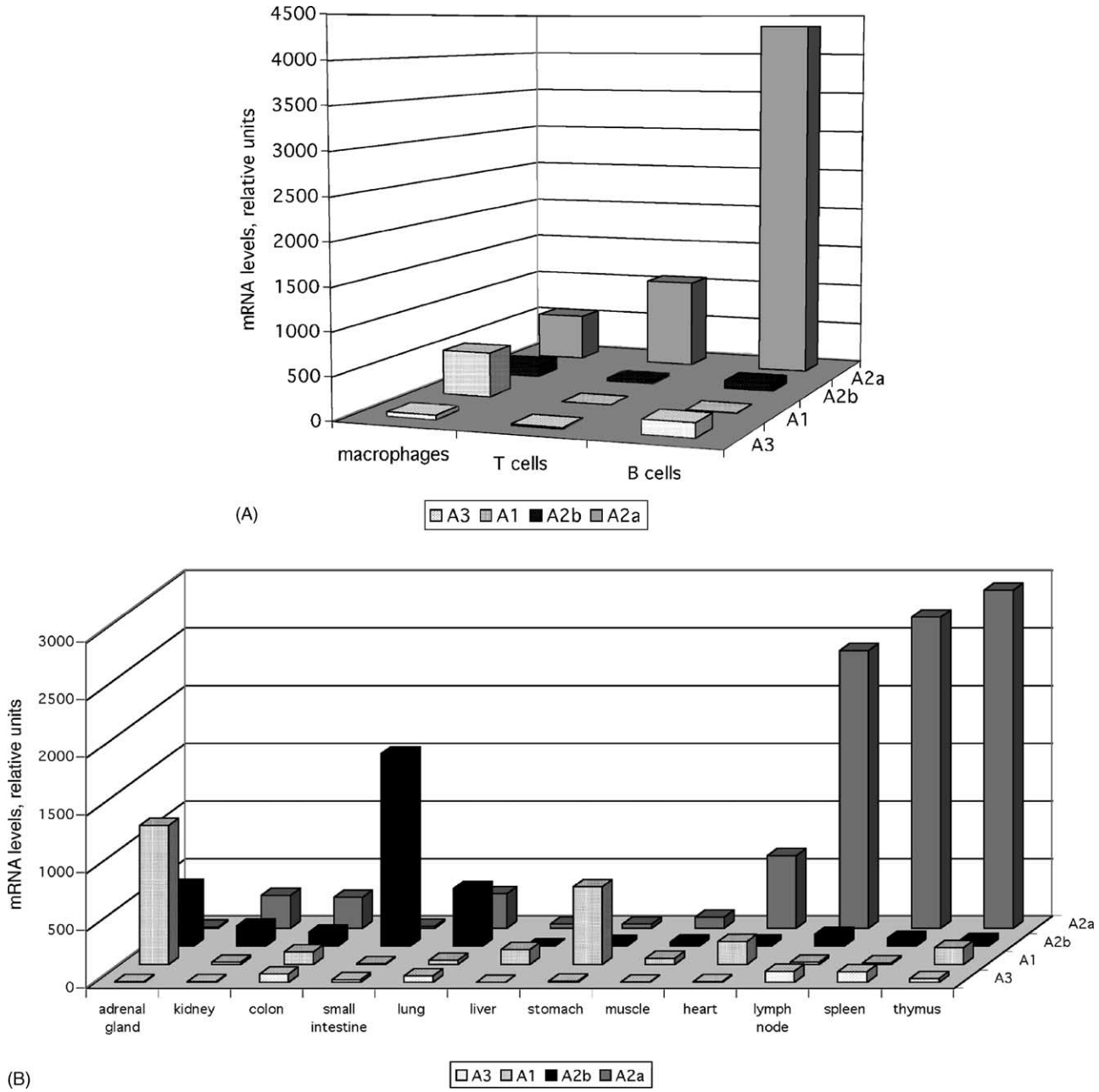


Fig. 1. (A) Comparison of the expression of Ado receptor mRNA in macrophages, T cells, and B cells from spleen. Macrophage, T cell, and B cell subsets were purified from the spleen of a wild-type mouse (N = 1) using immunomagnetic separation as described in "Section 2." (B) Comparison of the expression of Ado receptor mRNA in lymphoid and non-lymphoid organs. Total RNA was extracted from the organs of a wild-type mouse (N = 1). The data shown are representative of two experiments.

lysing buffer (Quality Biological Inc.) to remove red blood cells. Measurements of cAMP were performed in quadruplicates, as described previously [22]. Ado and CGS21680 [2-(4-((2-carboxymethyl)phenyl)ethylamino)-5′-*N*-ethylcarboxamidoadenosine] were purchased from Sigma, and ZM241385 [4-(2-(7-amino-2-(2-furyl)(1,2,4)-triazolo(2,3-*a*)(1,3,5)triazin-5-yl amino)ethyl)phenol] was obtained from Tocris.

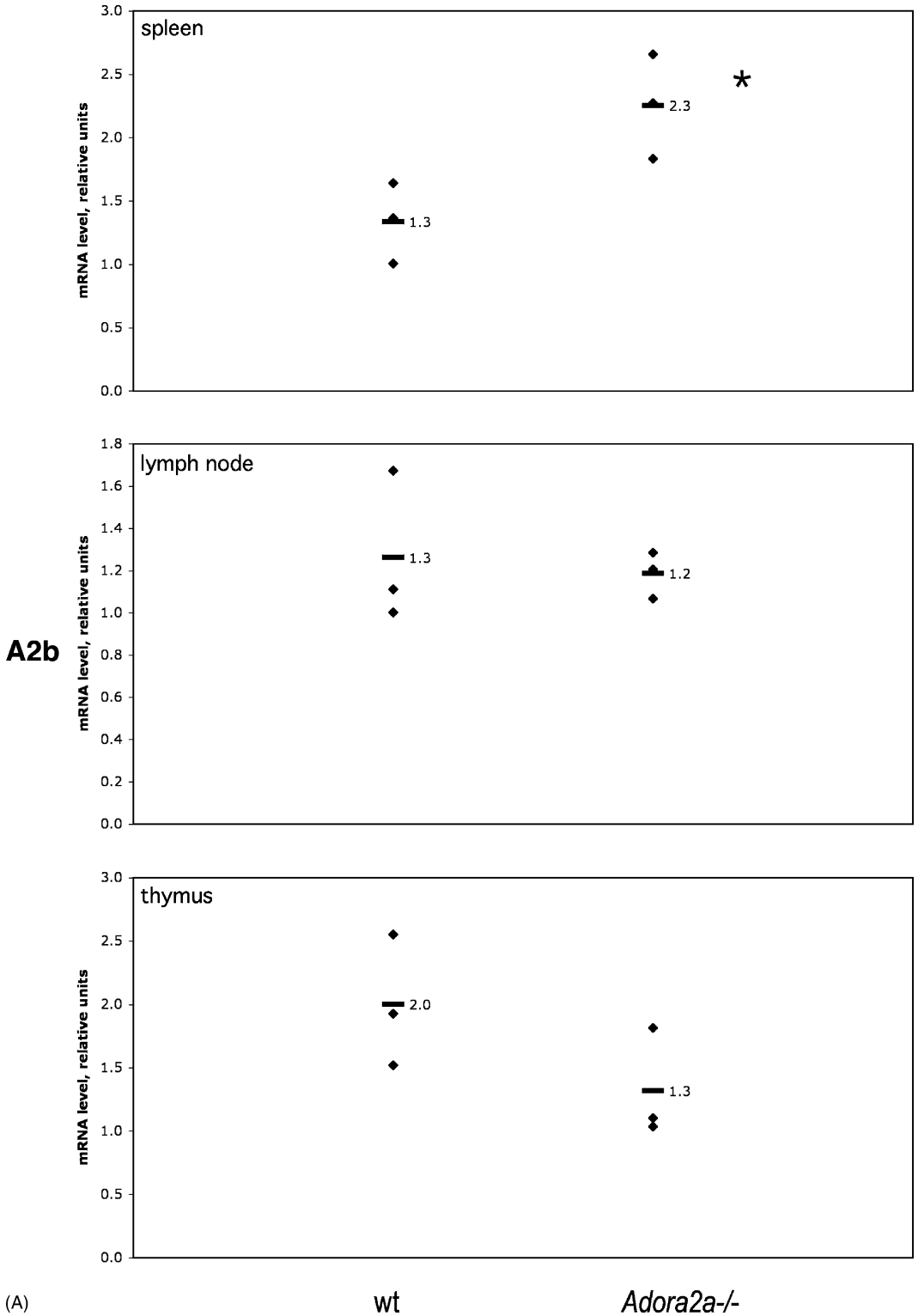


Fig. 2. Expression of A2b (A), A3 (B), and A1 (C) receptor mRNA in lymphoid organs from wild-type (N = 3) and *Adora2a*$^{-/-}$ mice (N = 3). Dots represent relative values for individual mice. Horizontal bars indicate the mean value between individual values. Differences between groups were evaluated using Student's *t*-test. Key: (*) $P < 0.05$, compared with wild-type mice.

3. Results and discussion

Real-time RT–PCR was used to evaluate the transcriptional levels of Ado receptors in cells from different lymphoid and non-lymphoid organs. Because the interpretation of studies of mRNA expression depends on standardization of samples and assays, the choice of the housekeeping gene is very important, and dictates the careful comparison of commonly used internal standards [23,24]. This was done by performing real-time RT–PCR with individual samples of GAPDH mRNA, β-actin mRNA, L32 mRNA, and 18S rRNA and comparing their expression ratios against total RNA, using incorporation of radioactive CTP in cDNA during reverse transcription. The

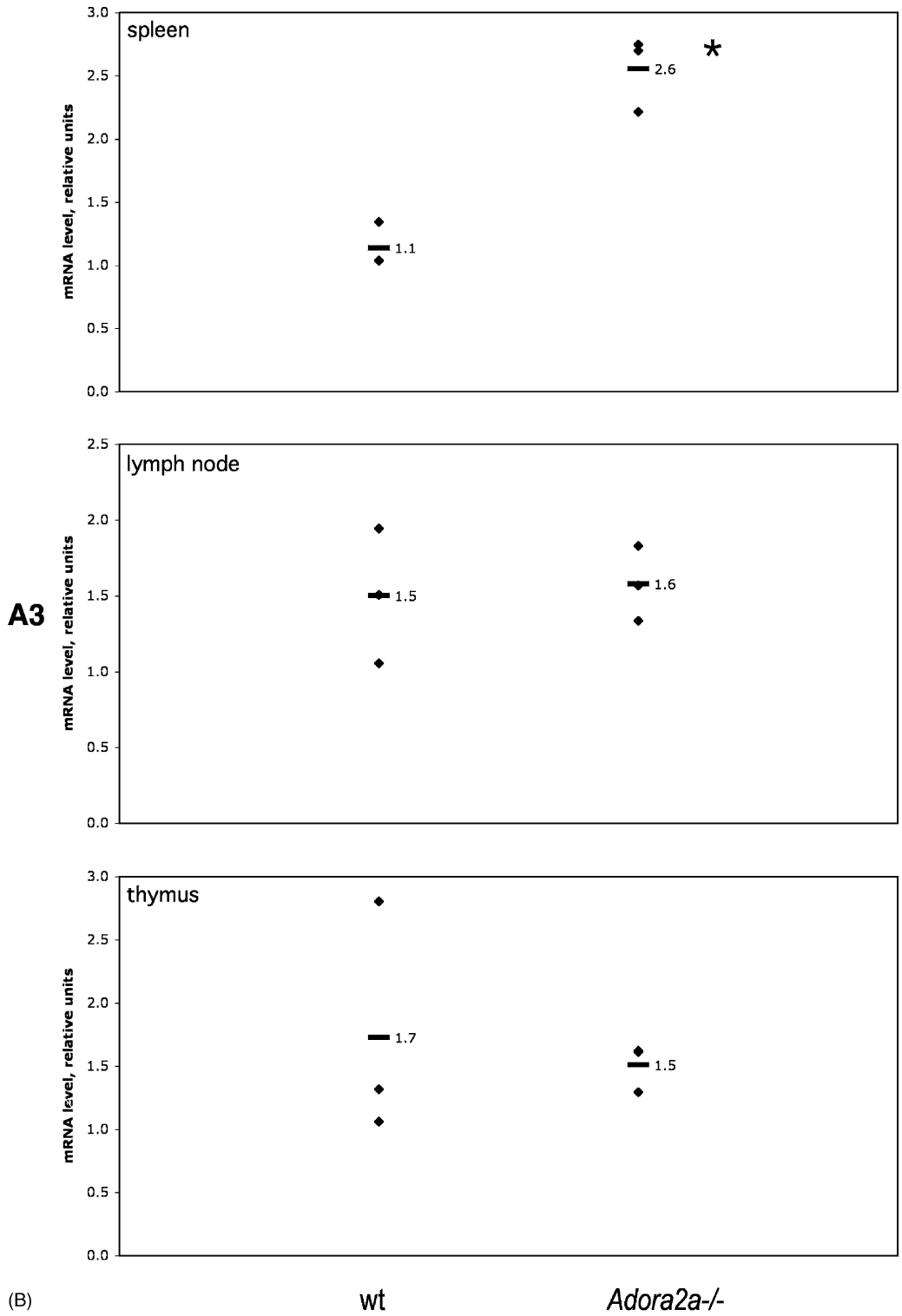


Fig. 2. (*Continued*)

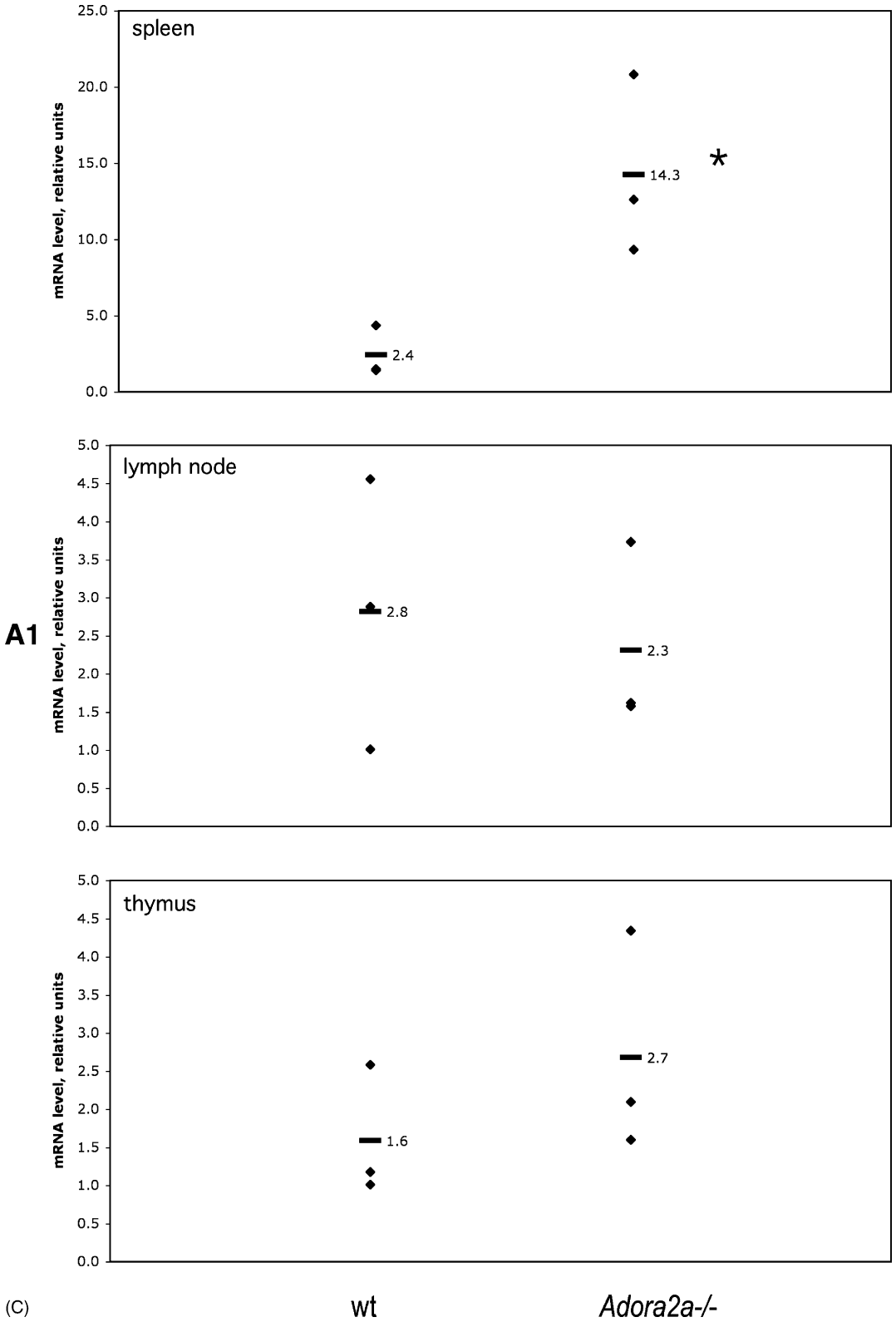


Fig. 2. (*Continued*).

results of these experiments showed that 18S rRNA appears to be the closest to an acceptable and reliable internal standard (data not shown).

Using real-time RT–PCR, we evaluated the expression of different Ado receptors in lymphoid and non-lymphoid tissues of wild-type mice. Figure 1 shows that B cells were found to express more A2a mRNA than T cells and macrophages. In general, lymphoid organs expressed high levels of A2a mRNA as compared with different non-lymphoid tissues (Fig. 1B).

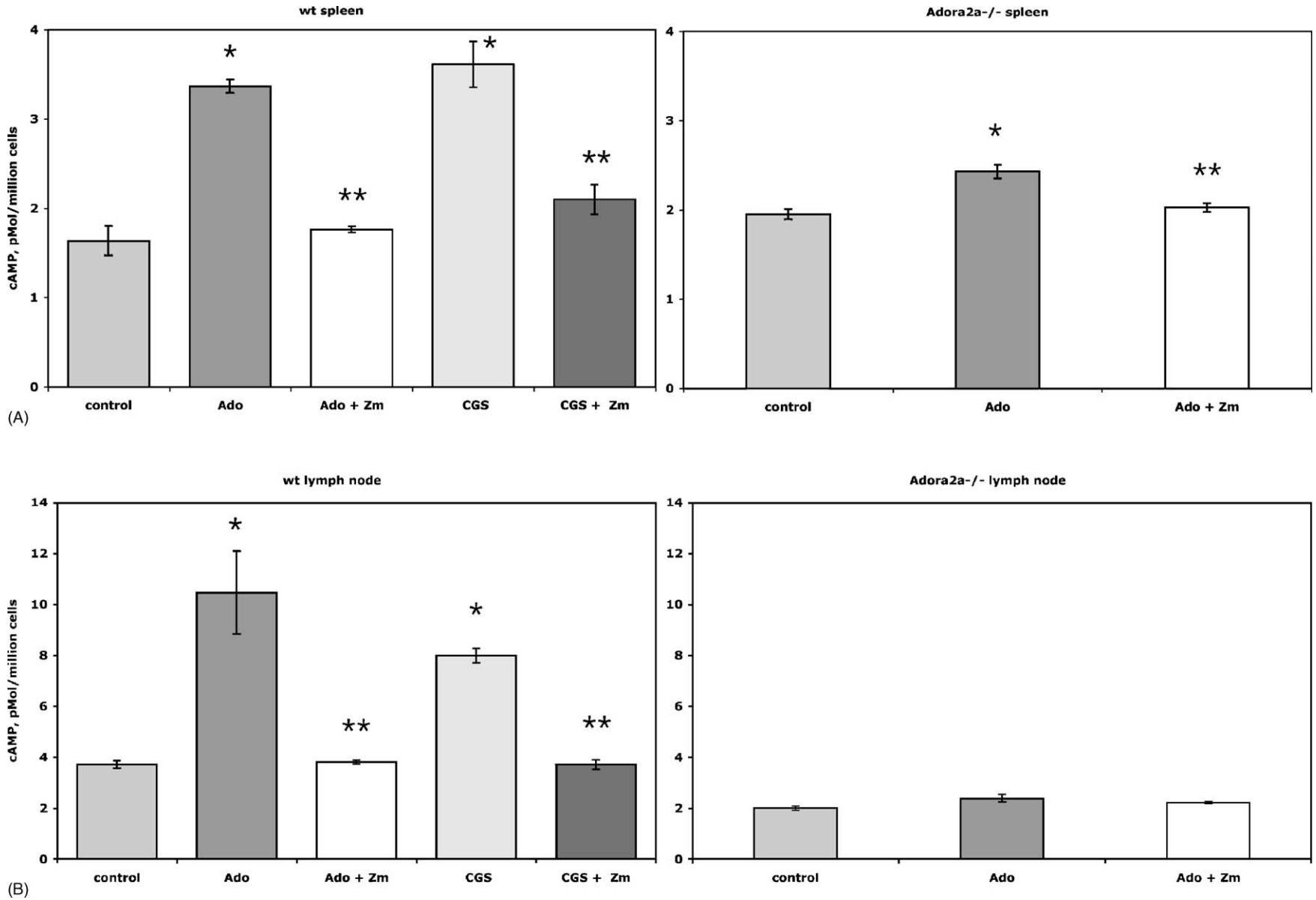
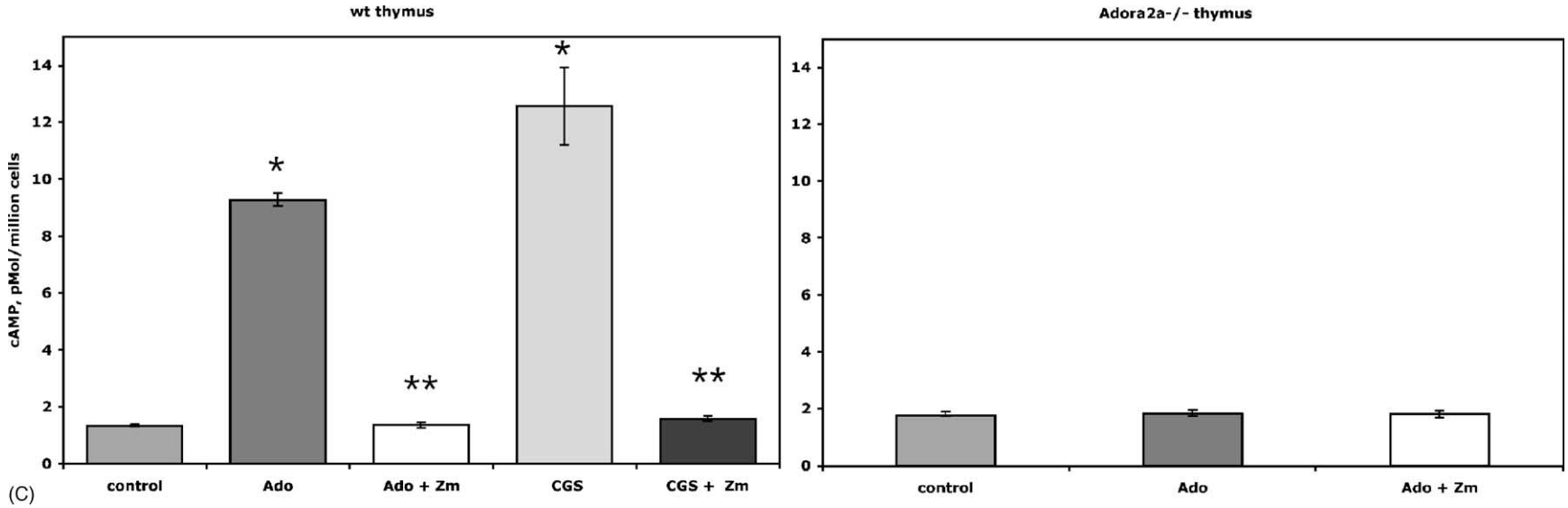


Fig. 3. Measurements of cAMP accumulation in splenocytes (A), lymph node cells (B), and thymocytes (C). Cells from a wild-type mouse (N = 1) and an *Adora2a*$^{-/-}$ mouse (N = 1) were incubated with either 50 μM Ado or 1 μM CGS21680 (CGS) in the presence of 1 μM ZM241385 (Zm), where indicated. cAMP measurements were performed in quadruplicate. Data are expressed as means ± SEM. Differences between groups were evaluated using Student's *t*-test. Key: (*) $P < 0.05$, compared with controls; and (**) $P < 0.05$, compared with cells incubated with Ado and CGS21680. The data shown were reproducible in four experiments.



(C)

Fig. 3. (*Continued*).

Next, we addressed the main question and compared the Ado receptor expression in lymphoid organs of normal and *Adora2a*$^{-/-}$ mice. Flow cytometric analysis of lymphoid organs demonstrated that proportions of macrophages, T cells, and B cells in spleen, lymph nodes, and thymus were not changed in *Adora2a*$^{-/-}$ mice as compared with wild-type littermates (data not shown). Figure 2 compares the mRNA expression levels of various Ado receptors between normal and age-matched *Adora2a*$^{-/-}$ mice. Of interest, the ~1.7 times increase in A2b receptor mRNA expression was observed only in the spleen and not in thymus or lymph nodes of *Adora2a*$^{-/-}$ mice (Fig. 2A). A3 receptor and A1 receptor mRNA levels were also higher in the spleens of *Adora2a*$^{-/-}$ mice but no changes were observed in lymph nodes and thymus (Fig. 2B and C, respectively).

To test whether the absence of A2a receptor is compensated for by other Ado receptors on a functional level, particularly by the cAMP-elevating A2b receptor, we measured cAMP levels in immune cells incubated with extracellular Ado. We show that *Adora2a*$^{-/-}$ lymphocytes from lymph nodes and thymus did not respond to Ado stimulation (Fig. 3), although a small increase in cAMP was detected in Ado-stimulated splenocytes (Fig. 3A). This cAMP elevation may reflect the increase in A2b receptor expression or the functioning of constitutively expressed A2b receptors. Further studies using A2b receptor-deficient mice should help in discriminating between these two possibilities. Wild-type lymphocytes produced cAMP when stimulated with Ado or CGS21680, an A2a receptor agonist, while ZM241385, an A2a/A2b antagonist, inhibited this induction of cAMP (Fig. 3). As *Adora2a*$^{-/-}$ lymphocytes were unable to produce sufficient amounts of cAMP after stimulation with high concentrations of Ado, which is capable of stimulating both A2a and A2b receptors, these data demonstrate that the absence of A2a receptors in lymphocytes is not compensated for by functional A2b receptors. Thus, the main finding of these studies is the inability of immune cells to significantly up-regulate functional cAMP-elevating A2b receptors in *Adora2a*$^{-/-}$ mice. However, the relatively small but consistently observed expression of cAMP-elevating A2b receptor and increase in A2b, A1, and A3 receptor mRNA was detected in the spleen, but not in other immune organs of *Adora2a*$^{-/-}$ mice. The reasons for this small up-regulation remain to be investigated, but it is clear that it is not of functional significance to be reflected in compensatory effects in *Adora2a*$^{-/-}$ mice. The data reported here may explain the dramatic results of genetic ablation or pharmacologic inactivation of A2a receptors [19], which caused run-away inflammatory tissue damage and prolonged accumulation of proinflammatory cytokines. Accordingly, the critical role of the A2a receptor in these processes is non-redundant and cannot be compensated for by up-regulation of other Ado receptors.

A very interesting issue remains as to whether A2b or A3 Ado receptors will be up-regulated on the surface of immune cells after chronic inflammation in *Adora2a*$^{-/-}$ mice. The experiments reported here, as well as earlier acute inflammation assays [19], were performed with naïve mice that did not experience the inflammatory insults, and no intracellular signaling that could affect the levels of other Ado receptors was observed. Although it remains to be conclusively proven, the presence of putative cAMP response elements on the A3 promoter region [25] raises the possibility that cAMP increases may lead to the up-regulation of A3 receptor expression. It is not clear, however, why the increase in A2b receptor mRNA expression in the spleen is accompanied by only a very small increase in Ado-induced cAMP levels (Fig. 3A). It will be especially interesting to clarify the reasons for this increase of A2b mRNA found in the spleen but not in the lymph nodes or the thymus. One plausible explanation for this could be the much larger proportion of B cells present in the spleen, in contrast to the lymph nodes and thymus.

These mRNA expression studies strongly suggest that the A2a receptor is the major functional Ado receptor that attenuates activation of immune cells. This conclusion is consistent with A2a receptors playing a key and non-redundant role in the down-regulation of earlier stages of acute inflammation [19].

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