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# 1,3,7-Trimethylxanthine (Caffeine) May Exacerbate Acute Inflammatory Liver Injury by Weakening the Physiological Immunosuppressive Mechanism<sup>1</sup>

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The genetic elimination of A2A adenosine receptors (A2AR) was shown to disengage the critical immunosuppressive mechanism and cause the dramatic exacerbation of acute inflammatory tissue damage by T cells and myeloid cells. This prompted the evaluation of the proinflammatory vs the anti-inflammatory effects of the widely consumed behavioral drug caffeine, as the psychoactive effects of caffeine are mediated largely by its antagonistic action on A2AR in the brain. Because caffeine has other biochemical targets besides A2AR, it was important to test whether the consumption of caffeine during an acute inflammation episode would lead to the exacerbation of immune-mediated tissue damage. We examined acute and chronic treatment with caffeine for its effects on acute liver inflammation. It is shown that caffeine at lower doses (10 and 20 mg/kg) strongly exacerbated acute liver damage and increased levels of proinflammatory cytokines. Because caffeine did not enhance liver damage in A2AR-deficient mice, we suggest that the potentiation of liver inflammation was mediated by interference with the A2AR-mediated tissue-protecting mechanism. In contrast, a high dose of caffeine (100 mg/kg) completely blocked both liver damage and proinflammatory cytokine responses through an A2AR-independent mechanism. Furthermore, caffeine administration exacerbated liver damage even when mice consumed caffeine chronically, although the extent of exacerbation was less than in "naive" mice that did not consume caffeine before. This study suggests an unappreciated "man-made" immunological pathogenesis whereby consumption of the food-, beverage-, and medication-derived adenosine receptor antagonists may modify an individual's inflammatory status and lead to excessive organ damage during acute inflammation. *The Journal of Immunology*, 2007, 179: 7431–7438.

Caffeine (1,3,7-trimethylxanthine) is one of the most popular habitually consumed psychostimulants. Effects of caffeine could be mediated by its action on several different targets such as cAMP phosphodiesterase, phosphatidylinositol-3 kinase, and adenosine receptors (1, 2). The behavioral activation by caffeine is largely accounted for by the antagonism of tonic activation of the A2A adenosine receptor (A2AR)<sup>3</sup> in the brain (1). A2AR is a G<sub>s</sub> protein-coupled receptor, and ligand binding to A2AR increases intracellular cAMP. Because immune cells express relatively high levels of A2AR (3), A2AR stimulation and the subsequent cAMP increase result in the inhibition of immune functions. Pharmacological stimulation by an A2AR agonist results in the inhibition of proliferation (4), cytokine production (5),

the cytotoxicity of T cells (6), and the activation of monocytes (7) and granulocytes (8). The anti-inflammatory effect of A2AR agonist has been demonstrated in inflammation in vivo, including ischemia/reperfusion injury (9, 10), airway inflammation (11), and T cell-dependent acute hepatitis (12).

The mechanism of physiologically relevant increases in the levels of endogenous adenosine to enable the adenosine-mediated down-regulation of inflammation in hypoxic areas of inflamed tissues is supported by observations of the obstruction of blood perfusion in inflamed tissue (13). Accordingly, the tissue hypoxia caused by limited blood supply may lead to an increase of local adenosine levels, probably because of insufficient ATP production and the inhibition of adenosine-metabolizing enzyme (14–16). Failure to produce a sufficient amount of ATP under hypoxia may result in the accumulation of its degradation product, adenosine. In addition, because hypoxia inhibits the activity of adenosine kinase (17, 18), the lack of adenosine removal accelerates the increase of local adenosine concentration. Although there are other classes of endogenous anti-inflammatory molecules (19), the adenosine-A2AR pathway was found to play a critical role in the physiological down-regulation of acute inflammation and the protection of tissues in vivo (12, 15). This was illustrated by observations of much increased inflammatory tissue damage in A2AR-deficient mice or wild-type mice treated with a selective A2AR antagonist (12).

These data raised the alarming possibility that caffeine, acting as an antagonist of A2AR, may inhibit an important A2AR-mediated tissue-protecting mechanism. This, in turn, suggested that caffeine might exacerbate tissue damage if consumed during an acute inflammation episode. However, caffeine is not a selective A2AR antagonist although its action as a psychostimulant is due to A2AR

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<sup>3</sup> Abbreviations used in this paper: A2AR, A2A adenosine receptor; ALT, alanine aminotransferase; A1R, A1 adenosine receptor; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine.

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antagonism in the brain (1). Caffeine does have properties other than being an A2AR antagonist. At higher concentrations, caffeine may act in vivo as an inhibitor of cAMP phosphodiesterase (1) and increase immunosuppressive cAMP content in immune cells.

To clarify this clinically and immunologically important issue, we examined the effects of caffeine on inflammation by using two distinct, widely used, and well-documented liver inflammation models: Con A-induced hepatitis and *Pseudomonas* exotoxin A-induced hepatitis. It was shown that i.v. injection of Con A induces T cell-dependent liver injury (20). The necroinflammatory liver damage accompanies the massive infiltration of inflammatory cells, including T lymphocytes, that resembles human viral/auto-immune hepatitis. Con A-induced liver injury has been studied extensively, and T cells, NKT cells (21, 22) and Kupffer cells (23) are found to be indispensable for the induction of liver damage. Furthermore, the participation of cytokines in liver damage has been also reported, i.e., IFN- $\gamma$  (24), TNF- $\alpha$  (25), and IL-4 (21, 22). For *Pseudomonas* exotoxin A-induced liver damage, i.v. administration of this bacterial toxin is shown to induce T cell-dependent liver injury (26, 27). The mechanism is suggested to involve the stimulation of T cell mitogenesis by *Pseudomonas* exotoxin A and the inhibition of protein synthesis, which sensitizes parenchymal hepatocytes to cytokine-induced cell death.

In this article we report the alarming proinflammatory effects of caffeine in vivo in mice with experimentally induced acute liver inflammation. This caffeine effect was mediated by inhibition of the tissue-protecting mechanism via A2AR. We also show that these effects of caffeine change dose dependently. Indeed, a high dose of caffeine suppressed liver damage by an A2AR-independent mechanism, most likely by the inhibition of cAMP-phosphodiesterase.

## Materials and Methods

### Reagents

Con A (type IV), *Pseudomonas* exotoxin A, D-galactosamine, and caffeine were purchased from Sigma-Aldrich. ZM241385 and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were from Tocris Bioscience. Serum alanine aminotransferase (ALT) levels were measured using a determination kit from Biotron Diagnostics.

### Mice

Female C57BL/6 mice were housed in the animal facility of the National Institute of Allergy and Infectious Diseases or Northeastern University (Boston, MA) and used at 7–9 wk of age. C57BL/6-background *A2AR*<sup>-/-</sup> and age-matched control (*A2AR*<sup>+/+</sup>) mice are obtained from parallel breeding in a colony of *A2AR*<sup>-/-</sup> mice. *A2AR*<sup>-/-</sup> mice were backcrossed 11 times to C57BL/6 mice (28). The mice were kept under a conventional 12-h light-dark cycle, and liver inflammation was induced during the light phase (11 a.m.). The animal experiment procedures were in accordance with institutional animal care guidelines.

### Analysis of blood caffeine levels by HPLC

Mice received an i.p. injection of caffeine (20 mg/kg) and either 30, 60, 90, or 120 min later a blood sample was obtained. The serum sample was assayed for caffeine and caffeine metabolites (theobromine, paraxanthine, and theophylline) by HPLC with UV detection (274 nm) as described by Frye et al. (29) using a Hewlett Packard HPLC system (model 1050). The internal standard was  $\beta$ -hydroxyethyl-theophylline. Retention times were 3.0 min for theobromine, 4.5 min for paraxanthine, 5.2 min for theophylline, 5.7 min for the internal standard, and 7.8 min for caffeine.

### Induction of liver inflammation by Con A

Female C57BL/6 mice were injected with Con A (12 mg/kg, i.v.) to induce liver inflammation (20). Adenosine receptor antagonists, including caffeine, were injected i.p. just before Con A injection. ZM241385 (2 mg/kg) and DPCPX (1.5 mg/kg) were dissolved in saline containing 2% DMSO and 2% Cremophor EL. Caffeine (10, 20, or 100 mg/kg) was dissolved in saline. D-Galactosamine (700 mg/kg) was given i.p. 15 min before Con A when needed. In a chronic consumption experiment, caffeine dissolved at

0.07% (w/v) was given as drinking water for 10 days before the induction of liver injury. The consumption of caffeinated water was 4 ml/mouse/day, and the volume was not different from the intake of plain water. The magnitude of liver damage was evaluated by serum transaminase levels and tissue histology. The liver tissue 24 h after Con A injection was fixed in 10% formalin-PBS and embedded in paraffin. H&E and TUNEL staining were performed by Molecular Histology. Serum IL-4, TNF- $\alpha$ , IFN- $\gamma$ , IL-12p35, and IL-10 levels were determined by ELISA (R&D Systems).

### *Pseudomonas* exotoxin A-induced liver injury

T cell-dependent liver damage was demonstrated in mice injected with *Pseudomonas* exotoxin A (26, 27). This model was originally established and has been studied extensively using the BALB/c strain. In this experiment, *Pseudomonas* exotoxin A (100  $\mu$ g/kg) was injected i.v. to female BALB/c mice. Some of the mice also received i.p. injection of caffeine (20 mg/kg) just before *Pseudomonas* exotoxin A. In addition, caffeine was given as drinking water (0.07%) to maintain caffeine levels. Serum ALT levels were measured after 12 h.

### RNase protection assay

Total RNA was extracted by solubilization in 1 ml of RNASTAT-60 (Tel-Test). RNA was dissolved in water, and 2  $\mu$ g of RNA were analyzed by an RNase protection assay using the Multi-Probe RNase Protection Assay System and the mCK-3b Multi-Probe Templates Set (BD Pharmingen) according to the manufacturer's instructions.

### cAMP assay

Peripheral blood was collected from the mice that received an i.p. injection of caffeine. The blood was immediately mixed with EDTA (final concentration 7.5 mM) and centrifuged for plasma. Plasma cAMP levels were determined by ELISA (Amersham Biosciences) according to instructions.

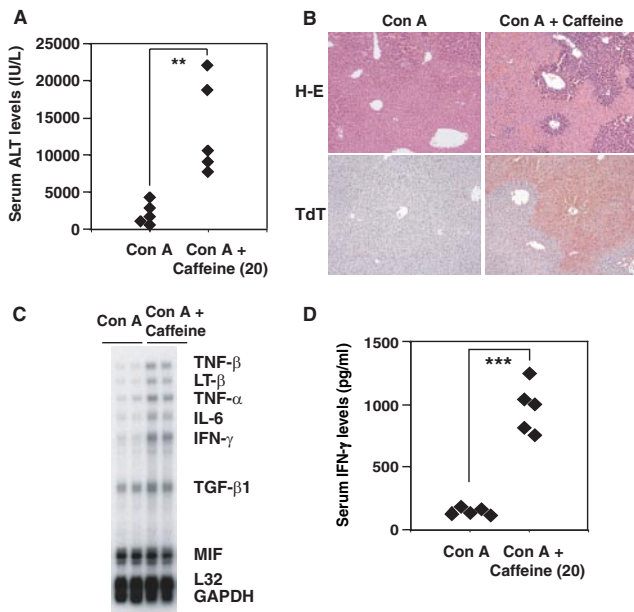
## Results

### Caffeine exacerbates acute hepatitis by antagonizing A2AR

Time-dependent change in serum caffeine concentrations after i.p. injection was analyzed using HPLC. We found that i.p. injection of caffeine into mice (20 mg of caffeine per kg of body weight) resulted in serum caffeine levels reaching 40  $\mu$ M after 30 min. Caffeine levels then declined with a half-life of 50 min, and this was associated with a rise in paraxanthine (data not shown). These levels of caffeine in serum are sufficiently high to antagonize adenosine signaling through the A2AR (1) on activated mouse immune cells.

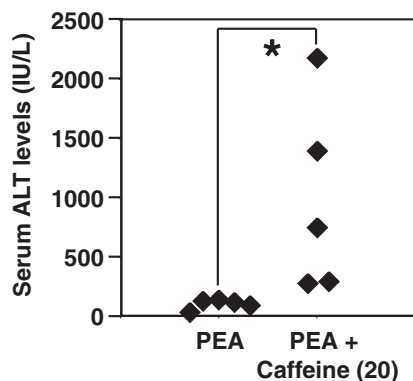
This dose of caffeine (20 mg/kg) coinjected with Con A significantly exacerbated acute liver injury in mice more than the injection of Con A alone. This was evidenced by increased levels of serum transaminase (Fig. 1A). The histochemical examination confirmed the caffeine-mediated exacerbation of inflammatory damage by observations of extensive tissue damage and greater infiltration of inflammatory cells into the livers of caffeine-treated mice (Fig. 1B). The extensive hepatocyte death after caffeine coinjection was also demonstrated by TUNEL staining (Fig. 1B). To test whether caffeine also affected the expression of proinflammatory cytokines, mRNA levels in the spleen were examined by an RNase protection assay. Fig. 1C demonstrates the increases of mRNA levels of cytokines (i.e., TNF- $\alpha$ , TNF- $\beta$ , lymphotoxin- $\beta$ , IL-6, and IFN- $\gamma$ ) in mice coinjected with caffeine. In accordance with mRNA levels, there was also an increase in the serum protein levels of proinflammatory cytokines such as IFN- $\gamma$  (Fig. 1D).

We next examined another hepatitis model to confirm the enhancement of inflammation by caffeine. Intravenous administration of *Pseudomonas* exotoxin A causes T cell-dependent acute liver damage (26, 27). As shown in Fig. 2, the coadministration of caffeine increased the acute liver damage induced by *Pseudomonas* exotoxin A. Thus, caffeine-induced exacerbation of inflammation was demonstrated by using two independent hepatitis models.

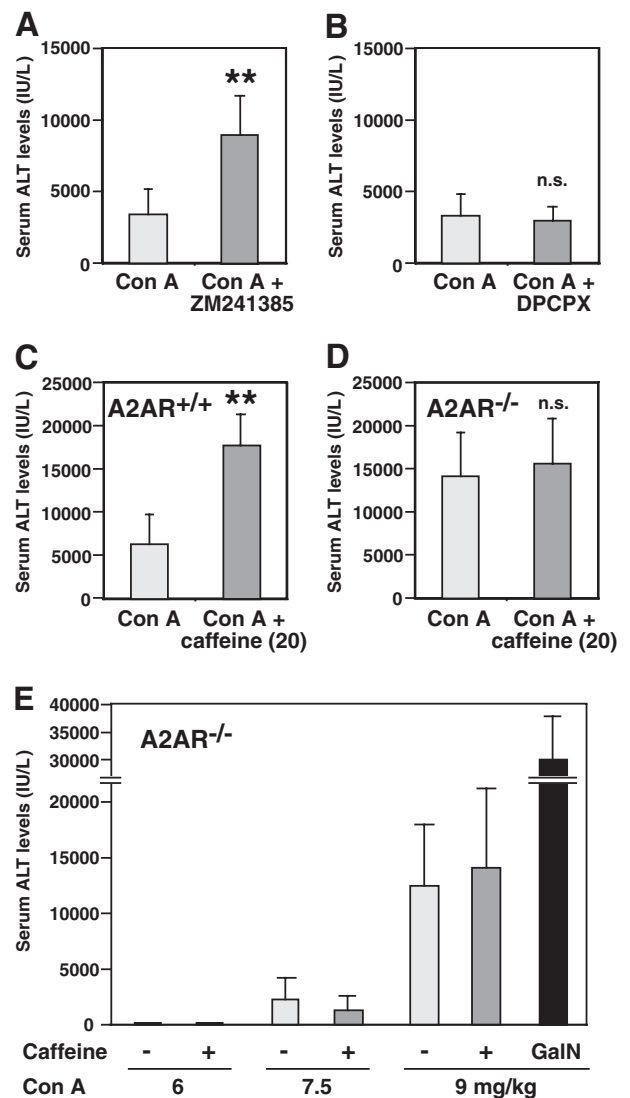


**FIGURE 1.** Effects of caffeine on liver damage in mice with acute hepatitis. *A*, Caffeine (20 mg/kg) exacerbated Con A (12 mg/kg, i.v.)-induced acute liver inflammation. Serum transaminase levels were measured after 8 h (five mice per group). ALT levels in untreated and caffeine alone groups were  $26 \pm 6$  and  $30 \pm 11$  IU/L, respectively. The data represent four separate experiments. *B*, Histochemical evidence for the more severe liver inflammation in caffeine-treated mice. The liver tissue was examined by hematoxylin and eosin (H-E) and TUNEL (TdT) assays showing liver degeneration and extensive cell death. *C* and *D*, Expression of proinflammatory cytokines in caffeine-treated mice is higher than that in mice treated with Con A alone. mRNA expression in the spleen after 1.5 h (*C*) was analyzed by RNase protection assay (LT- $\beta$ , lymphotoxin- $\beta$ ; MIF, migration inhibitory factor). The data shown here represent three similar results. Serum IFN- $\gamma$  levels after 8 h (*D*) were determined by ELISA (five mice per group). Levels of IFN- $\gamma$  in untreated and caffeine alone groups were under the detection limit. The statistical significance was calculated by Student's *t* test: \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Because caffeine can nonspecifically antagonize both A1 and A2 adenosine receptors (1), caffeine-induced exacerbation of liver damage was compared with the effects of subtype-selective antagonists for the A1 and A2A adenosine receptors (A1R and A2AR, respectively) (30). Corresponding with a previous report (12), the



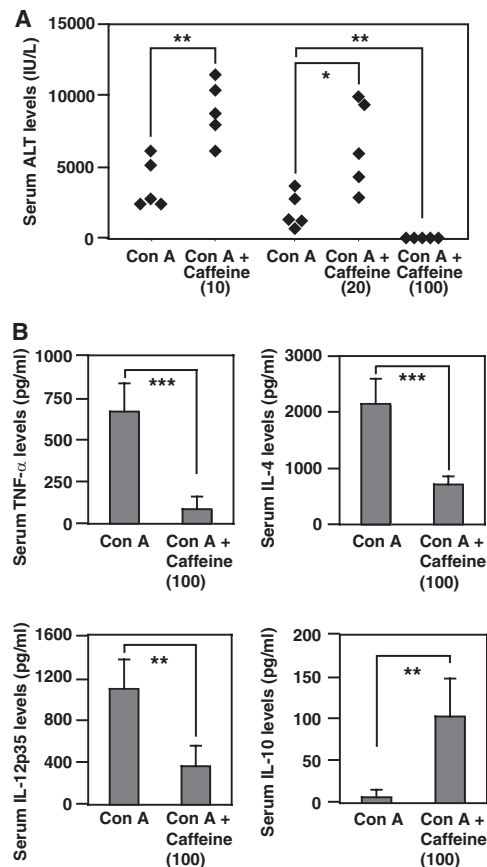
**FIGURE 2.** Exacerbation of *Pseudomonas* exotoxin A (PEA)-induced liver damage by caffeine. Caffeine (20 mg/kg) was injected along with *Pseudomonas* exotoxin A (100  $\mu$ g/kg). Serum transaminase levels at 12 h are shown (five mice per group). The data represent three separate experiments. The statistical significance was calculated by Student's *t* test: \*,  $p < 0.05$ .



**FIGURE 3.** Caffeine exacerbates liver damage through the antagonism of A2AR. *A* and *B*, Inflammatory liver damage was exacerbated by blocking A2AR but not A1R. Acute liver injury was induced in wild-type mice as described in Fig. 1. The A2AR-selective antagonist ZM241385 (*A*) or the A1R-selective antagonist DPCPX (*B*) was injected just before Con A administration. Serum transaminase levels at 8 h are shown (5 mice per group). ALT levels in untreated mice and mice that received DPCPX alone or ZM241385 alone were  $33 \pm 12$ ,  $32 \pm 16$  and  $34 \pm 22$  IU/L, respectively. The data represent three separate experiments. *C* and *D*, Caffeine did not enhance liver damage in A2AR-deficient mice. The dose of Con A was 12 mg/kg for wild-type mice (*C*,  $n = 5$ ) and 9 mg/kg for A2AR-deficient mice (*D*,  $n = 5$ ). ALT levels in untreated A2AR<sup>+/+</sup> mice and A2AR<sup>-/-</sup> mice were  $28 \pm 15$  and  $28 \pm 12$  IU/L, respectively. *E*, A2AR-deficient mice also received different doses of Con A for the induction of various degrees of liver damage. Caffeine (20 mg/kg) still failed to enhance the damage, although cotreatment with D-galactosamine (GalN) could exacerbate the liver damage ( $n = 4$ ). The statistical significance was calculated by Student's *t* test: \*\*,  $p < 0.01$ ; "n.s." means not significant.

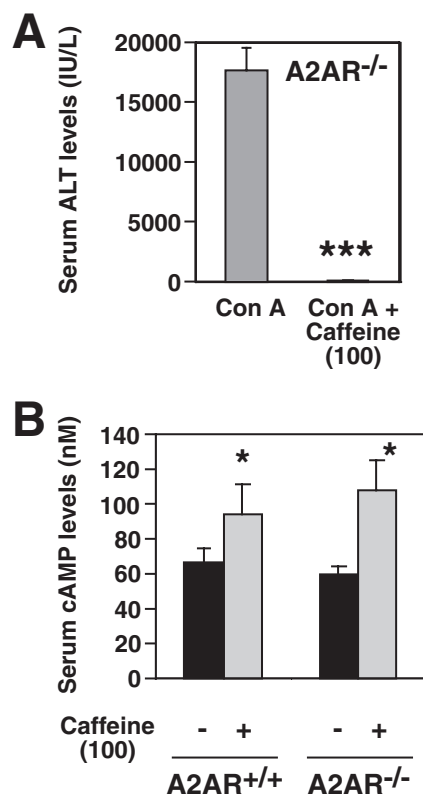
pharmacological inhibition of A2AR by ZM241385 significantly exacerbated Con A-induced liver injury (Fig. 3*A*). However, the A1R-selective antagonist DPCPX did not increase the liver damage (Fig. 3*B*). These data suggest that antagonism of A2AR, but not of A1R, by caffeine may result in pronounced liver damage.

To confirm the participation of the A2AR-dependent mechanism in the exacerbation of liver damage by caffeine, we examined



**FIGURE 4.** Dose-dependent change of effects of caffeine on acute liver damage. *A*, Lower doses of caffeine (10 and 20 mg/kg) potentiate acute liver inflammation whereas a higher dose of caffeine (100 mg/kg) blocks Con A-induced liver inflammation. Serum aminotransferase levels were measured after 8 h (five mice per group). Control ALT levels for the first set of experiment were  $36 \pm 8$  and  $40 \pm 16$  IU/L in untreated mice and the mice that received 10 mg/kg of caffeine alone, respectively. ALT levels in untreated mice and mice that received either 20 or 100 mg/kg caffeine were  $31 \pm 12$ ,  $28 \pm 11$ , and  $29 \pm 10$  IU/L, respectively. *B*, Effects of a high dose of caffeine (100 mg/kg) on cytokine levels (five mice per group). Serum IL-4, TNF- $\alpha$ , and IL-10 levels were measured after 1.5 h. IL-12p35 levels were measured in sera collected 3 h after Con A injection. Levels of cytokines in the untreated and the caffeine alone groups were under the detection limit. The data represent three separate experiments. The statistical significance was calculated by Student's *t* test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

the effect of caffeine in A2AR-deficient mice. As expected, caffeine did not enhance acute liver damage in A2AR-deficient mice, whereas in a parallel experiment the same dose of caffeine strongly increased liver damage in wild-type (A2AR<sup>+/+</sup>) mice (Fig. 3, *C* and *D*). Although the deficiency of A2AR by itself strongly promotes liver damage (12), the lack of caffeine-mediated exacerbation in A2AR-deficient mice was not because of the induction of maximum liver damage by Con A alone. In fact, while caffeine did not enhance the liver damage in A2AR-deficient mice, D-galactosamine could further exacerbate it (Fig. 3*E*). In addition, we also tested lower doses of Con A for the induction of weaker liver damage, which will allow a chance for further exacerbation in A2AR-knockout mice. Doses at 7.5 and 6 mg/kg resulted in intermediate (ALT: 3,500 IU/L) and minimum (ALT: 100 IU/L) liver damage in A2AR-knockout mice, respectively. However, co-treatment with caffeine did not exacerbate liver damage in A2AR-knockout mice no matter what dose of Con A was injected (Fig.

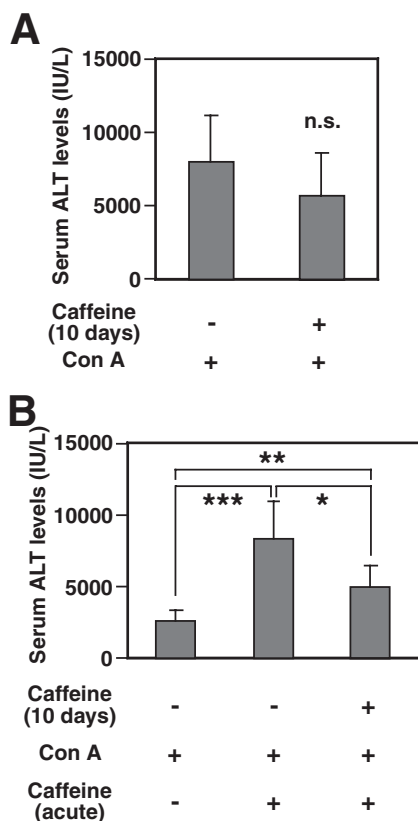


**FIGURE 5.** Immunosuppression by a high dose of caffeine. *A*, A high dose of caffeine (100 mg/kg) inhibited liver injury by an A2AR-independent mechanism. A2AR-deficient mice (five mice per group) received an optimal dose of Con A (10 mg/kg, i.v.) and a high dose of caffeine (100 mg/kg, i.p.) at the same time. Serum ALT levels were determined after 8 h. *B*, Early cAMP induction by a high dose of caffeine. Wild-type C57BL/6 mice and A2AR-deficient mice (four mice per group) received caffeine (100 mg/kg, i.p.), and plasma cAMP levels after 30 min were determined by ELISA. The statistical significance was calculated by Student's *t* test: \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

3*E*). These results show that caffeine can enhance acute hepatitis through the inhibition of A2AR-mediated cellular events.

#### Protection from hepatitis by a high dose of caffeine

A2AR is a G<sub>s</sub>-coupled receptor that induces intracellular cAMP upon ligand binding, and the increase of cAMP levels results in immunosuppression. The interference of adenosine binding to A2AR by caffeine can inhibit the adenosine-mediated increase of cAMP levels. However, at high concentrations caffeine is known to act as an inhibitor of cAMP phosphodiesterase (1). The inhibition of phosphodiesterase activity may result in the accumulation of cAMP and inhibition of inflammatory responses. Therefore, we tested different doses of caffeine in the induction of liver inflammation. As shown in Fig. 4*A*, caffeine as low as 10 mg/kg still exacerbated liver damage as was observed after the injection of 20 mg/kg. However, the injection of a high dose of caffeine (100 mg/kg) provided strong protection against acute liver damage. Injection of a high dose of caffeine also resulted in decreases of serum TNF- $\alpha$ , IL-4, and IL-12p35 levels and an increase of the IL-10 level (Fig. 4*B*). These results show that the effect of caffeine on inflammation is biphasically dependent on doses. Namely, lower doses of caffeine enhanced proinflammatory cytokine induction and tissue destruction, while a high dose of caffeine suppressed tissue damage by the inhibition of proinflammatory cytokine responses and the induction of an anti-inflammatory cytokine, IL-10.



**FIGURE 6.** Acute caffeine administration is proinflammatory even in chronic caffeine consumers. *A*, Mice consumed 0.07% caffeine in drinking water for 10 days and received Con A. *B*, The caffeine administered to mice that chronically consumed caffeine is less proinflammatory than when it was given to “naive” mice. The effect of caffeine in mice given with 0.07% caffeinated water for 10 days was compared with a group that did not receive caffeinated drinking water (“naive”). Serum aminotransferase levels were measured after 8 h (five mice per group). ALT levels in untreated mice and mice that received either acute or chronic caffeine alone were  $38 \pm 10$ ,  $41 \pm 5$ , and  $33 \pm 15$  IU/L, respectively. The data represent five separate experiments. The statistical significance was calculated by Student's *t* test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; “n.s.” means not significant.

The suppression of liver damage by a high dose of caffeine was not dependent on the A2AR-mediated mechanism. Indeed, when a high dose of caffeine was injected into A2AR-deficient mice it again completely inhibited the induction of acute liver damage (Fig. 5A). This suggests that high concentrations of caffeine activate an A2AR-independent immunosuppressive mechanism that can overcome immunoenhancement by the antagonism of A2AR. The immunosuppression by cAMP may explain the mechanism, because the increase of plasma cAMP levels was observed after the injection of this dose of caffeine in wild-type mice as well as in A2AR-deficient mice (Fig. 5B).

#### Effect of chronic caffeine consumption

Although we have examined the acute administration of caffeine to clarify its effects on inflammation, consumption of caffeine in humans is chronic in most cases. To test how habitual consumption affects inflammation, caffeine was given to mice as drinking water for 10 days before triggering acute liver inflammation. Then, we assessed the extent of liver damage after the injection of Con A and the effect of a low dose of caffeine. The control experiment showed that chronic caffeine consumption history alone did not augment the liver damage (Fig. 6A). When Con A was coinjected

with an acute dose of caffeine, the liver damage was enhanced even after chronic caffeine consumption (Fig. 6B). The comparison between habitual caffeine consumer mice and control naive mice revealed that this chronic caffeine pretreatment reduced the ability of acute caffeine administration to exacerbate the acute liver damage (Fig. 6B).

#### Discussion

The identification of the adenosine-A2AR pathway as an important physiological down-regulatory mechanism of inflammatory responses (12, 15) prompted us to examine naturally abundant compounds that can be antagonists of A2AR. Among this class of compounds, caffeine is the most widely consumed compound principally from beverages. We found that the injection of caffeine significantly increased liver damage in T cell-dependent hepatitis models (Figs. 1 and 2). The same dose of caffeine could also enhance liver damage when administered orally (data not shown). The increase of liver damage by caffeine could be explained by uninhibited proinflammatory responses. By acting as an A2AR antagonist, caffeine might have disabled the adenosine-mediated anti-inflammatory mechanism (1, 12). In agreement with this mechanism of action, there was the enhanced expression of proinflammatory cytokines in mice coinjected with caffeine (Fig. 1, C and D).

These effects of caffeine are similar to the potentiation of hepatitis observed in mice treated with a selective antagonist of A2AR (12, 15). However, caffeine is not a specific antagonist for A2AR; caffeine can antagonize A1R as well as A2AR, whereas it has much lower affinity to the A3 adenosine receptor (1). To distinguish roles of A1R and A2AR in liver damage, we compared the effects of a selective A1R antagonist (DPCPX) with those of an A2AR antagonist (ZM241385) (30). In contrast to the exacerbation of liver damage by ZM241385, DPCPX did not enhance Con A-induced liver injury at all (Fig. 3). Con A-induced liver damage was not increased even when the dose of DPCPX was increased up to 7.5 mg/kg (data not shown). This suggests that the antagonism of A1R is not likely a mechanism for the enhancement of hepatic inflammation by caffeine. As was shown by using ZM241385 or A2AR-deficient mice, adenosine signaling through A2AR is playing an important role in the protection of tissues from the overactivation of immune responses. The induction of liver injury in A2AR-deficient mice confirmed the inability of a low dose of caffeine to enhance acute liver damage in the absence of A2AR (Fig. 3). These results indicate inhibition of the tissue-protecting adenosine-A2AR pathway as a mechanism of the exaggeration of tissue damage by the administration of caffeine during ongoing acute inflammation.

In contrast to these results, caffeine was also reported to block liver inflammation (31). We confirmed in dose-response studies that high doses of caffeine could affect acute hepatitis in an opposite direction from the proinflammatory effect observed with low doses of caffeine. A high dose of caffeine strongly suppressed liver damage and activated an anti-inflammatory mechanism at an early stage of hepatitis induction. The inhibition of proinflammatory cytokines (Fig. 4B) may explain the decrease of liver damage, because early inductions of TNF- $\alpha$  (25), IL-4 (21, 22) and IL-12 (32) are essential in the pathogenesis of Con A-induced liver injury. In addition, the induction of an anti-inflammatory cytokine IL-10 (Fig. 4B) is known to be protective in the tested model (33).

Indeed, we found that caffeine could have opposite (proinflammatory vs anti-inflammatory) effects on liver inflammation, with the dose of caffeine determining the overall effect on acute liver

inflammation. This can be explained not only by caffeine's properties as an A2AR antagonist but also as a cAMP phosphodiesterase inhibitor (1). As an A2AR antagonist caffeine inhibits intracellular cAMP induction, but when acting as a phosphodiesterase inhibitor it enhances immunosuppressive cAMP accumulation by inhibiting its degradation. The anti-inflammatory effect of caffeine at a high dose was also demonstrated in A2AR-deficient mice (Fig. 5A), suggesting that A2AR is not involved in this immunosuppression. The increase of plasma cAMP levels after a high dose of caffeine in both wild-type and A2AR-deficient mice (Fig. 5B) corresponds to this speculation. When caffeine acts as a phosphodiesterase inhibitor, it requires an ~20 times higher concentration than as an A2AR antagonist (1). Therefore, the anti-inflammatory effects appearing only after the injection of higher dose of caffeine are most likely due to the inhibition of cAMP phosphodiesterase (1, 34), although we do not exclude the possible contribution of the inhibition of phosphatidylinositol-3-kinase (2) and/or other mechanisms.

Caffeine consumption is typically chronic in humans. It was, therefore, interesting to examine whether the chronic consumption of caffeine affects the outcome of inflammation. In contrast to an acute administration, chronic consumption of caffeinated water alone (without an acute dose) failed to augment the tissue damage (Fig. 6A). This difference may be explained by the actual presence or absence of caffeine when inflammation is induced. Because caffeine was given ad libitum in drinking water, caffeine levels in vivo are expected to change during the day depending on the drinking behavior of the mice. Mice are nocturnal; therefore, caffeine concentrations in vivo may have dropped to the level that is not sufficient to antagonize A2AR when we induced liver inflammation during the daytime (11 a.m.). This speculation is supported by the induction of liver inflammation at night (11 p.m.) while the mice are drinking caffeinated water, when caffeine drinking alone (without an acute dose) could strongly exacerbate the liver damage (ALT levels: Con A alone,  $1520 \pm 905$ ; Con A plus caffeinated water,  $9337 \pm 2541$  IU/L). These results suggest that inflammatory tissue damage is enhanced only in the actual presence of caffeine, which provides the inhibition of an A2AR-mediated down-regulating mechanism of inflammation.

Next, we examined acute caffeine administration to mice that have chronically consumed caffeine, and we found that caffeine injection exacerbated liver damage even in the chronic caffeine consumers (Fig. 6B). This result suggests that the actual presence of caffeine can enhance tissue-damaging inflammatory responses regardless of a previous history of caffeine exposure, although the enhancement of liver damage in chronic caffeine consumers was not as strong as in caffeine-naïve mice. Correspondingly, pretreatment with chronic caffeine intake alone even tended to decrease the damage, although the difference was not statistically significant (Fig. 6A). These results indicate that the effects of caffeine on the immune system may be subject to the development of tolerance, just as tolerance to caffeine develops in the CNS (1, 35, 36). The up-regulation of adenosine receptors, including A2AR, after long-term caffeine treatment (37) may have increased sensitivity to the anti-inflammatory action of adenosine and somewhat reduced the liver damage. Chronic caffeine consumption was also shown to protect the brain from ischemic damage (38, 39); however, changes in the number of adenosine receptors are not likely the cause of tissue protection in these models (40).

Although we show that low vs high doses of caffeine modify inflammation in an opposite direction, we would suggest that the potentiation of inflammation by low doses of caffeine may

be clinically more relevant because the majority of human coffee drinkers habitually consume lower, "proinflammatory" doses of caffeine. In our experiment, serum caffeine concentration after the injection of a low dose of caffeine (20 mg/kg) was 40  $\mu$ M at 30 min and decreased at a half-life of 50 min. Of clinical importance, the metabolism and clearance of caffeine is significantly slower in humans, i.e., the half-life of caffeine is 3–4.5 h in humans vs <1 h in mice (1). Therefore, the same per kilogram dose of caffeine does not reproduce the same blood concentration in humans and mice. Indeed, the serum caffeine concentration after a dose of 2 mg/kg was 10  $\mu$ M in mice while it was 25  $\mu$ M in humans (41, 42). Considering such differences, it is suggested that 10 mg/kg of caffeine in rodents represents 3.5 mg/kg in humans, which corresponds to 2–3 cups of coffee for a 70-kg human (1). The amount of caffeine in a single cup of coffee (100 mg) for humans is also estimated to be equivalent to 5 mg/kg in rodents. The exacerbation of liver damage was demonstrated in mice receiving 10 mg/kg (Fig. 4A), but not with 5 mg/kg (data not shown). Although a single cup of coffee may not significantly enhance inflammatory responses, it should be noted that 2–3 cups of coffee may enhance inflammation in human. It is also interesting that caffeine is detected in habitual caffeine-consuming humans at concentrations capable of antagonizing A2AR, 10–50  $\mu$ M in moderate to heavy caffeine consumers (43, 44). Other studies showed that when humans acutely ingested 2–2.5 mg/kg caffeine (corresponding to the amount in two cups of coffee), blood caffeine concentration was ~20  $\mu$ M (41, 45, 46). In contrast, amounts of caffeine much higher than what people usually consume will be required for the immunosuppressive effects of caffeine. Because normal caffeine consuming habits of humans can be sufficient to block A2AR, our data may have clinical relevance.

The accumulating epidemiological studies strongly suggest the inverse relation between coffee consumption and chronic liver diseases including cirrhosis (47–53). The relative risk of cirrhosis decreased in coffee drinkers dependent on the amount of coffee consumption. The proinflammatory effects of caffeine described here seem to be controversial with these epidemiological data. However, the participation of caffeine in the hepatoprotection provided by coffee is not clear from epidemiological studies. Tea and other caffeine-containing beverages, unlike coffee, were reported not to have an inverse relation to the risk of cirrhosis (47, 50). In contrast, a recent study suggested an inverse relation of tea intake, as well as coffee intake, with incidences of chronic liver diseases (53). The mechanism for the beneficial effects of coffee on chronic liver inflammation in humans (47–53) may be different from the described novel tissue-damaging effects of caffeine during acute inflammation. Enhancement of inflammation is detrimental to inflammatory disorders; however, in contrast, it could be beneficial when the enforcement of immune responses is favorable, i.e., immune responses against tumor and infectious agents. Recently, we demonstrated that inhibition of the adenosine-A2AR pathway might be a promising target for the promotion of antitumor immune responses (54). In that study, caffeine was beneficial in enhancing T cell-mediated antitumor responses. Therefore, it is also possible that the consumption of caffeine promoted antiviral immune responses to decrease the virus titer in hepatitis patients. Thus, caffeine may strongly influence inflammatory processes but, in addition to the dose and duration of caffeine administration (Figs. 4 and 6), there is a possibility that the effect of caffeine may be different depending on the type and stage of the inflammatory process.

In summary, acting as A2AR antagonist, caffeine consumption may enhance acute liver inflammation. We suggest a possibility that caffeine might worsen the acute phase of viral hepatitis and drug-induced hepatic damage (i.e., acetaminophen hepatotoxicity). Future clinical and epidemiological studies may be necessary to determine whether the use of caffeine should be influenced by the knowledge of an individual's status of inflammation. It remains to be established whether caffeine is similar to A2AR antagonists in the exacerbation of not only acute liver inflammation but also of chronic hepatitis and inflammation in other tissues, including lung inflammation and sepsis (12, 55). It is also appealing to consider the A2AR-antagonizing effects of caffeine in developing novel adjuvants for cancer vaccines and vaccines against pathogens.

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## Disclosures

The authors have no financial conflict of interest.

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