

Plasma glutamate–modulated interaction of A_{2A}R and mGluR5 on BMDCs aggravates traumatic brain injury–induced acute lung injury

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The bone marrow–derived cell (BMDC)–associated inflammatory response plays a key role in the development of acute lung injury (ALI). Activation of adenosine A_{2A} receptor (A_{2A}R) is generally considered to be antiinflammatory, inhibiting BMDC activities to protect against ALI. However, in the present study, we found that in a mouse model of neurogenic ALI induced by severe traumatic brain injury (TBI), BMDC A_{2A}R exerted a proinflammatory effect, aggravating lung damage. This is in contrast to the antiinflammatory effect observed in the mouse oleic acid–induced ALI model (a nonneurogenic ALI model.) Moreover, the A_{2A}R agonist CGS21680 aggravated, whereas the antagonist ZM241385 attenuated, the severe TBI–induced lung inflammatory damage in mice. Further investigation of white blood cells isolated from patients or mouse TBI models and of cultured human or mouse neutrophils demonstrated that elevated plasma glutamate after severe TBI induced interaction between A_{2A}R and the metabotropic glutamate receptor 5 (mGluR5) to increase phospholipase C–protein kinase C signaling, which mediated the proinflammatory effect of A_{2A}R. These results are in striking contrast to the well-known antiinflammatory and protective role of A_{2A}R in nonneurogenic ALI and indicate different therapeutic strategies should be used for nonneurogenic and neurogenic ALI treatment when targeting A_{2A}R.

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Abbreviation used: A_{2A}R, adenosine A_{2A} receptor; AC, adenylyl cyclase; ALI, acute lung injury; BMT, BM transplantation; GPCR, G protein–coupled receptor; IP, immunoprecipitation; MPEP, 2-methyl-6-(phenylethynyl)pyridine; PLC, phospholipase C; TBI, traumatic brain injury.

Traumatic brain injury (TBI) is a life-threatening syndrome that can be complicated by the dysfunction of peripheral organs such as lung, liver, kidney, or intestine. Among these, the association between TBI and subsequent acute lung injury (ALI) has been increasingly recognized (Dettbarn and Davidson, 1989; Bratton and Davis, 1997; Contant et al., 2001; Bronchard et al., 2004; Mascia et al., 2008). Mackersie et al. (1983) reported that 9 of 18 comatose victims with isolated TBI developed pulmonary edema, defined as increased extravascular lung fluid content measured by thermal green dye. Two studies have reported that 20–25% of patients with isolated TBI developed respiratory insufficiency (Fulton and Jones, 1975; Bratton and Davis, 1997). Moreover, Holland et al. (2003) investigated 137 patients with TBI and found

that ~31% developed ALI. In fact, TBI-induced ALI and its development may not only influence the lung epithelium, but may also impair brain function, aggravate the neurogenic injury, and cause higher mortality and worse prognosis. Although there is evidence to suggest that inflammation is the key pathological mechanism in TBI-induced ALI (Kalsotra et al., 2007; Jin et al., 2009), as it is in nonneurogenic ALI (such as ALI induced by lung trauma, shock, and sepsis; Wheeler and Bernard, 2007; Parekh et al., 2011; Qian et al., 2012), it is unclear how TBI triggers the lung inflammatory response.

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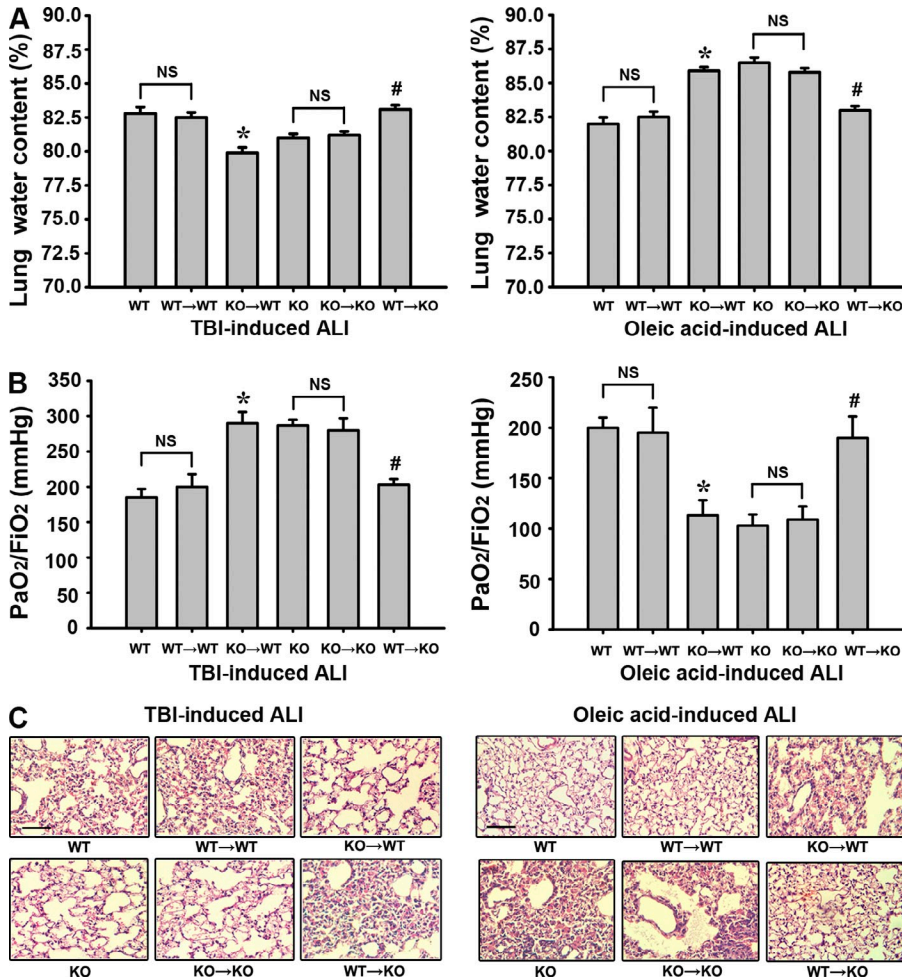


Figure 1. BMDC A_{2A}R differentially regulates the lung injury parameters in the severe TBI-induced ALI model and oleic acid-induced ALI model. At 24 h after injury, lung injury parameters were assessed. WT: WT mice; KO: global A_{2A}R KO mice; WT→WT: WT recipient mice with BMDCs from WT mice; KO→KO: KO recipient mice with BMDCs from KO mice; KO→WT: WT recipient mice with BMDCs from KO mice (selective inactivation of A_{2A}R in BMDCs of WT mice); WT→KO: KO recipient mice with BMDCs from WT mice (selective reconstitution of A_{2A}R in BMDCs of A_{2A}R KO mice). (A) The lung water content was assayed by the wet-dry method. (B) Blood gas analyses of arterial blood. (C) H&E staining for lung sections. Bars, 100 μm. (A and B) Data are expressed as mean ± SEM. *, P < 0.01, compared with the WT group; #, P < 0.01 compared with the KO group; NS, no significant difference between the two groups. For every parameter measured, n = 8–10 in each group, and each experiment was repeated three times.

BMDCs, including neutrophils, lymphocytes, monocytes, and eosinophils (also called WBCs), are the critical response cells for progression of inflammation in ALI/acute respiratory distress syndrome (Abraham, 2003; Nakajima et al., 2010; Grommes and Soehnlein, 2011). Adenosine A_{2A} receptor (A_{2A}R), one of four G protein-coupled adenosine receptors (A₁R, A_{2A}R, A_{2B}R, and A₃R), is found to be expressed on BMDCs and can regulate the function of BMDCs in several pathological conditions. Previous studies in multiple nonneurogenic ALI animal models such as LPS-induced ALI, lung ischemia-reperfusion injury, and lung injury in laparotomy-induced hemorrhagic shock have shown that activation of A_{2A}R plays an antiinflammatory role via inhibition of BMDC activities (Thiel et al., 2005; Haskó et al., 2006; Reutershan et al., 2007; Sharma et al., 2010). Accordingly, this receptor is considered an attractive potential target for therapeutic approaches to human ALI (Schepp and Reutershan, 2008). Conversely, in TBI and some other central nervous system injury models, A_{2A}R on BMDCs has been found to promote the inflammation of brain or spinal cord (Yu et al., 2004; Dai et al., 2010a). This leads us to speculate that in severe TBI-induced ALI (a neurogenic ALI), the role of BMDC A_{2A}R

may be different from that in nonneurogenic ALI and may be involved in the progression of TBI-induced ALI.

To confirm this hypothesis, we created BM chimeras to determine the role of BMDC A_{2A}R in a mouse model of severe TBI-induced ALI, comparing it with the oleic acid-induced ALI model (a nonneurogenic model). In human and mouse WBCs and neutrophils, the major components of BMDCs and the key reactive cells in ALI, we further investigated the mechanisms of BMDC A_{2A}R effects on inflammation associated with TBI-induced ALI.

RESULTS

Selective inactivation of BMDC A_{2A}R aggravates lung damage in the oleic acid-induced ALI model but exerts a protective effect in the severe TBI-induced ALI model

In the severe TBI-induced ALI model, we found that selective inactivation of BMDC A_{2A}R (KO→WT) significantly reduced lung water content (Fig. 1 A), elevated PaO₂/FIO₂ (P/F) ratios (Fig. 1 B), and attenuated histological signs of pulmonary injury (Fig. 1 C) at 24 h after injury when compared with WT littermates, consistent with the results observed in global A_{2A}R KO mice. Conversely, in the oleic

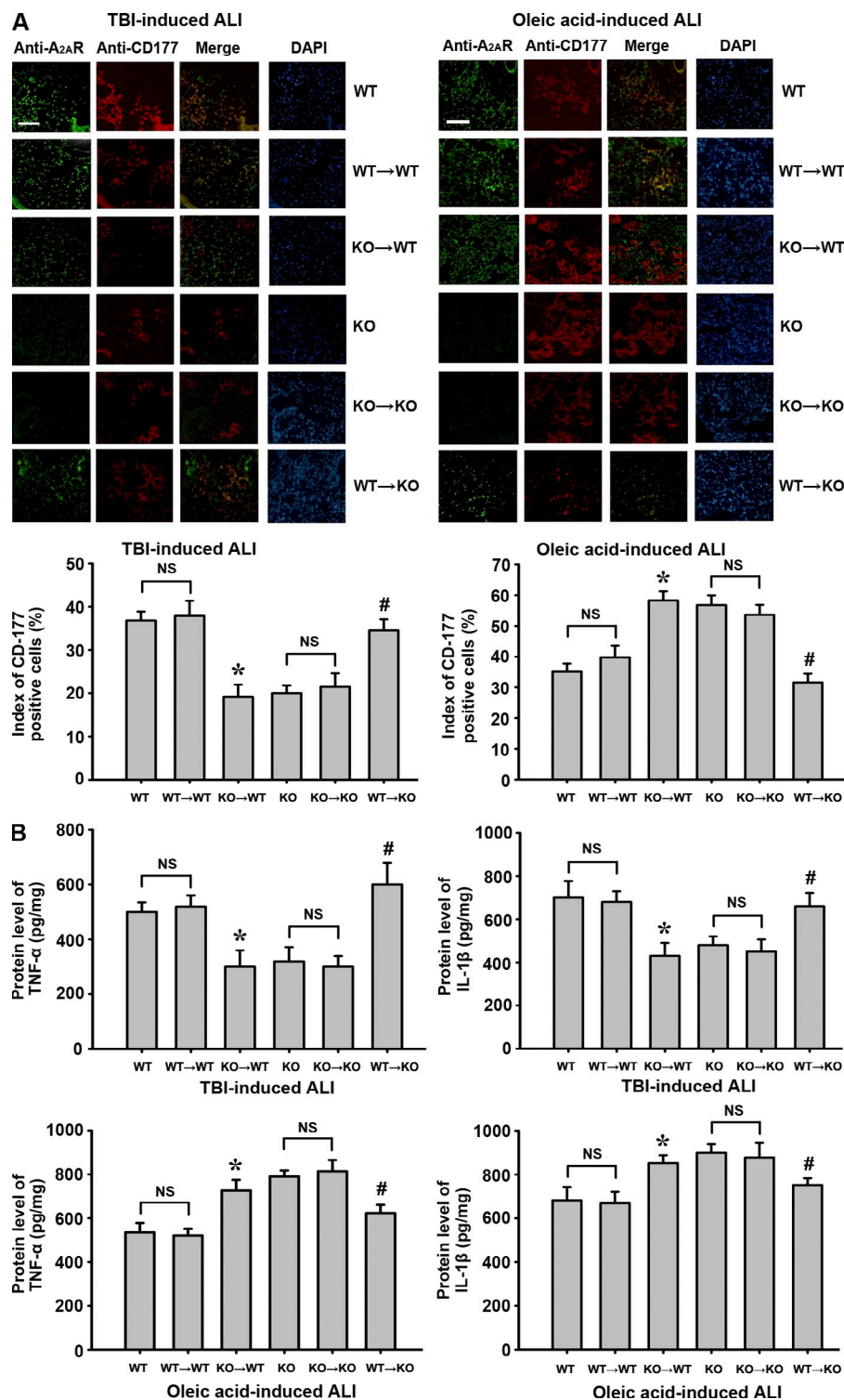


Figure 2. BMDC A_{2A}R inversely modulates the neutrophil infiltration and inflammatory cytokine expression in the severe TBI- and oleic acid-induced ALI models. (A) Alterations of neutrophil infiltration and A_{2A}R expression on neutrophils in lung tissues. Neutrophil counting and statistical analysis were performed by Image-Pro plus version 4.5 and presented as an index of CD-177-positive cells. Bars, 100 μ m. (B) Alterations of protein expression of TNF and IL-1 β in lung tissues. Values are expressed as mean \pm SEM of three independent experiments performed in triplicate. *, $P < 0.01$, compared with the WT group; #, $P < 0.01$ compared with the KO group; NS, no significant difference between the two groups; $n = 8-10$ for all groups. Each experiment was repeated three times.

acid-induced ALI model, each of these measures of lung damage was exacerbated in the mice with selective inactivation of BMDC A_{2A}R or global A_{2A}R KO (Fig. 1). Reconstitution of BMDC A_{2A}R in global A_{2A}R KO mice (WT→KO) eliminated the A_{2A}R KO-triggered protective effects in TBI-induced ALI and the deleterious effects in oleic

acid-induced ALI. In addition, no significant difference was observed between WT mice and WT mice transplanted with WT BMDCs (WT→WT) or between KO and KO→KO transplanted mice, excluding the possibility that these results were induced nonspecifically by BM transplantation (BMT; Fig. 1). These data strongly suggest that in contrast

to the protective effect of BMDC $A_{2A}R$ in oleic acid-induced ALI, BMDC $A_{2A}R$ plays a deleterious role in TBI-induced ALI.

BMDC-selective $A_{2A}R$ deficiency promotes inflammation in mice with oleic acid-induced ALI and suppresses inflammation in mice with severe TBI-induced ALI

As shown in Fig. 2 A, at 24 h after TBI, global $A_{2A}R$ KO mice and mice with selective inactivation of BMDC $A_{2A}R$ (KO \rightarrow WT) displayed reduced numbers of pulmonary neutrophils for TBI-induced ALI but enhanced numbers for oleic acid-induced ALI. Similarly, global or BMDC-specific $A_{2A}R$ KO mice showed increased pulmonary levels of the proinflammatory cytokines TNF and IL-1 β after TBI-induced ALI and reduced levels after oleic acid-induced TBI (Fig. 2 B). Reconstitution of BMDC $A_{2A}R$ in global $A_{2A}R$ KO mice (WT \rightarrow KO) eliminated the $A_{2A}R$ KO-triggered antiinflammatory effect in TBI-induced ALI and the proinflammatory effect in oleic acid-induced ALI (Fig. 2). These results indicate that BMDC $A_{2A}R$ plays a significant proinflammatory role in the progression of TBI-induced ALI.

Plasma glutamate level is increased more in severe TBI-induced ALI than in nonneurogenic ALI

In both the TBI- and oleic acid-induced ALI models, we measured plasma glutamate levels and found that they were much higher in mice with severe TBI-induced ALI (25–50 $\mu\text{mol/l}$) than in mice with oleic acid-induced ALI (8–15 $\mu\text{mol/l}$) at 6, 12, and 24 h after injury, even though the plasma glutamate level was elevated in both models (Fig. 3 A). To corroborate this, we also assayed the changes in plasma glutamate levels in patients suffering from TBI-induced ALI or lung trauma-induced ALI, which is also a nonneurogenic ALI. Consistent with the results in the animal models, the plasma glutamate concentrations of patients suffering from severe TBI-induced ALI were much higher than those of patients with lung trauma-induced ALI at 6, 12, and 24 h after injury (Fig. 3 B).

$A_{2A}R$ -metabotropic glutamate receptor 5 (mGluR5) interaction in BMDCs is detected in severe TBI-induced ALI rather than in nonneurogenic ALI

Glutamate has been previously reported to regulate $A_{2A}R$ activity in brain injury, and heteromeric complexes of adenosine receptors and mGluRs were found in the brain under physiological or pathological conditions (Ciruela et al., 2001; Ferré et al., 2002; Nishi et al., 2003; Dai et al., 2010b). However, only mGluR5 and mGluR2/3 have been reported to be expressed on white blood inflammatory cells (Gill and Pulido, 2001; Liu et al., 2010b). Accordingly, using specific antibodies for $A_{2A}R$, mGluR5, and mGluR2/3, we performed coimmunoprecipitation (co-IP) experiment to investigate the interaction between $A_{2A}R$ and these mGluRs. From WBC extracts of mice with TBI-induced ALI, the mouse antibody against $A_{2A}R$ coimmunoprecipitated a band corresponding to mGluR5 (Fig. 4 A). This band did not appear in immunoprecipitates from the cells of mice with oleic acid-induced ALI

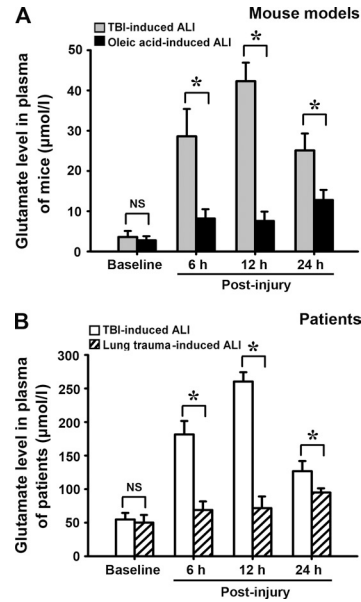


Figure 3. Plasma glutamate levels in severe TBI-induced ALI and nonneurogenic ALI over time. Plasma glutamate levels in mouse models and patients were detected at 6, 12, and 24 h after injury by HPLC. (A) Changes of plasma glutamate levels in mouse models (TBI- vs. oleic acid-induced ALI model). (B) Changes of plasma glutamate levels in patients (TBI- vs. lung trauma-induced ALI patients). Values are expressed as mean \pm SEM, and each experiment was repeated three times. *, $P < 0.01$ between the two groups; NS, no significant difference between the two groups. 10 patients with TBI-induced ALI and 8 patients with lung trauma-induced ALI were investigated. The number of mice for the assay at each time point was 8–10 in each group.

(Fig. 4 A). No IP of mGluR2/3 was obtained from WBCs in either of the two mouse ALI models (Fig. 4 A). Consistent with these results in animal models, analysis by SDS-PAGE and immunoblotting with anti-mGluR5 antibodies of immunoprecipitates obtained with human anti- $A_{2A}R$ showed a band corresponding to mGluR5 in WBCs of TBI-induced ALI patients but not those of lung trauma-induced ALI patients (Fig. 4 B). No mGluR2/3 band was detected in WBCs of either group (Fig. 4 A). In addition, using anti-mGluR5 antibody as the immunoprecipitating antibody and anti- $A_{2A}R$ antibody as the immunoblotting antibody, the interaction of $A_{2A}R$ and mGluR5 was confirmed in the WBCs from mice and patients (Fig. 4, C and D). These results indicate that $A_{2A}R$ -mGluR5 interaction is induced in BMDCs in severe TBI-induced ALI.

Elevation of plasma glutamate level and interaction of $A_{2A}R$ and mGluR5 in BMDCs are also observed in a mouse model of moderate TBI-induced ALI

To confirm the links between plasma glutamate level, $A_{2A}R$ -mGluR5 interaction in BMDCs, and TBI-induced lung damage, we used a mouse model of moderate TBI. Of 40 experimental mice with moderate TBI, 38 displayed decreased plasma glutamate levels at 6 h after TBI and recovered almost to baseline at 12 h after injury, which did not exhibit ALI

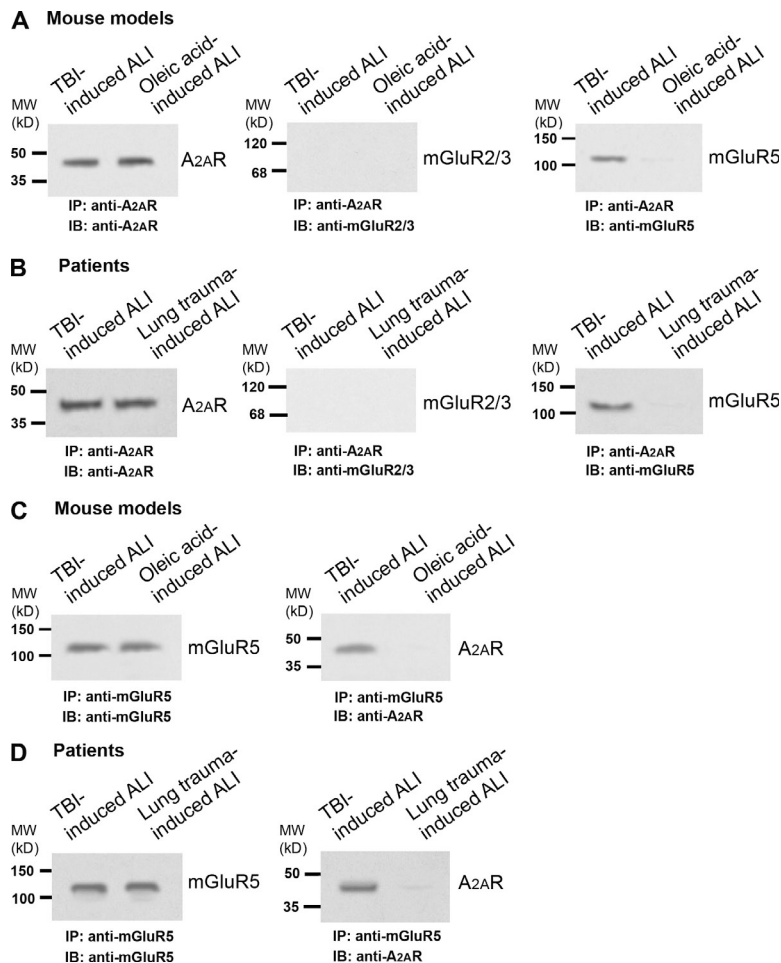


Figure 4. A_{2A}R–mGluR5 interaction is detected in WBCs of severe TBI-induced ALI mouse model or severe TBI-induced ALI patients. (A) Co-IP of A_{2A}R and mGluR2/3 or mGluR5 in WBCs of mouse models using anti-A_{2A}R antibody as the immunoprecipitating antibody. (B) Co-IP of A_{2A}R and mGluR2/3 or mGluR5 in WBCs of patients using anti-A_{2A}R antibody as the immunoprecipitating antibody. (C and D) Interaction of A_{2A}R and mGluR5 in WBCs of mouse models (C) or of patients (D) was confirmed by co-IP using anti-mGluR5 antibody as the immunoprecipitating antibody. Each experiment was repeated three times. IB, immunoblot.

after moderate TBI. Only two mice displayed increased plasma glutamate levels after injury (Fig. 5 A). At 24 h after TBI, only these two mice suffered from ALI with increased lung water content (Fig. 5 B), pathological changes in lung tissues (Fig. 5 D), and deficiency of pulmonary gas exchange (P/F ratio was below 300 mmHg; Fig. 5 C). Moreover, A_{2A}R–mGluR5 interaction in WBCs was observed only in these two mice (Fig. 5, E and F).

Elevated glutamate concentration induces A_{2A}R–mGluR5 interaction to promote the proinflammatory effect of A_{2A}R in neutrophils

To explore whether the elevated plasma glutamate level in TBI-induced ALI is involved in inducing the A_{2A}R–mGluR5 interaction and whether this interaction mediates the proinflammatory effect of BMDC A_{2A}R, we investigated the receptor interaction and the induction of proinflammatory cytokines in human and mouse neutrophils *in vitro* in the presence of varying concentrations of glutamate (the given glutamate concentrations were relative to the plasma glutamate levels *in vivo* described above). In mouse neutrophils in the presence of 30 and 300 $\mu\text{mol/l}$ glutamate, a band corresponding to mGluR5 was coimmunoprecipitated by anti-A_{2A}R antibodies (Fig. 6 A), and an A_{2A}R band was coimmunoprecipitated by anti-mGluR5

antibodies (Fig. 6 B), indicating the interaction between these receptors at these glutamate concentrations. At lower concentrations of glutamate, no co-IP was observed (Fig. 6, A and B). mGluR5 siRNA significantly decreased the expression of mGluR5 in mouse neutrophils, establishing our ability to knock down mGluR5 (Fig. 6 C). As shown in Fig. 6 D, the A_{2A}R agonist CGS21680 inhibited LPS-induced IL-1 β mRNA expression in neutrophils from WT mice in control medium and in the presence of 5 $\mu\text{mol/l}$ glutamate but enhanced IL-1 β mRNA levels in the presence of 30 and 300 $\mu\text{mol/l}$ glutamate (Fig. 6 D). This glutamate concentration-dependent change was not observed in neutrophils from A_{2A}R KO mice or when mGluR5 was knocked down by mGluR5 siRNA in WT neutrophils (Fig. 6 D).

Similar results were obtained in human neutrophils: in the presence of 300 $\mu\text{mol/l}$ glutamate (similar to the plasma glutamate level in patients with TBI-induced ALI), an mGluR5 band was coimmunoprecipitated by anti-A_{2A}R antibody (Fig. 6 E) and an A_{2A}R band was coimmunoprecipitated by anti-mGluR5 antibody (Fig. 6 F). And no co-IP was observed at lower concentrations of glutamate (Fig. 6, E and F). Moreover, CGS21680 switched from inhibiting (in the presence of 0, 5, or 30 $\mu\text{mol/l}$ glutamate) to promoting (at 300 $\mu\text{mol/l}$ glutamate) LPS-induced IL-1 β mRNA expression in human

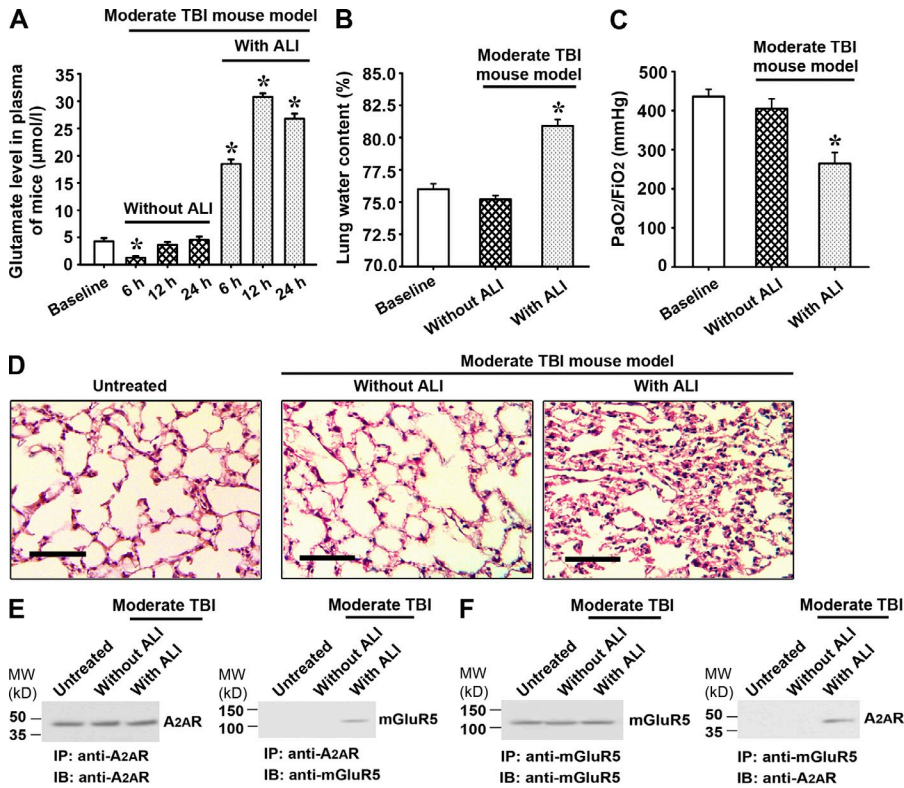


Figure 5. Plasma glutamate level, lung injury parameters, and WBC $A_{2A}R$ -mGluR5 interaction in moderate TBI mouse model. Moderate TBI was induced in 40 mice via cortical impact with the weight-dropping method. Only two of these mice were diagnosed with ALI at 24 h after TBI. (A) Changes of plasma glutamate levels at 6, 12, and 24 h after moderate TBI. (B) Lung water content. (C) Blood gas analyses of arterial blood. (A–C) Values are expressed as mean \pm SEM. (D) H&E staining for lung sections. Bars, 100 μ m. (E) Co-IP of $A_{2A}R$ and mGluR5 in WBCs using anti- $A_{2A}R$ antibody as the immunoprecipitating antibody. (F) Co-IP of $A_{2A}R$ and mGluR5 in WBCs using anti-mGluR5 antibody as the immunoprecipitating antibody. IB, immunoblot. *, $P < 0.01$ when compared with the baseline. Each experiment was repeated three times.

neutrophils, dependent on glutamate concentration (Fig. 6 G). The antiinflammatory effect of CGS21680 in the presence of 0, 5, and 30 μ mol/l glutamate was completely blocked by the selective $A_{2A}R$ antagonist ZM241385, whereas the proinflammatory effect of CGS21680 in the presence of 300 μ mol/l glutamate was suppressed to some extent by ZM241385 or mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) alone but completely suppressed by a combination of ZM241385 and MPEP (Fig. 6 G). These findings suggest that the increased plasma glutamate level caused by TBI-induced ALI promotes the interaction between $A_{2A}R$ and mGluR5 in BMDCs and that this interaction is critical for the proinflammatory effect exerted by activation of BMDC $A_{2A}R$.

Phospholipase C (PLC)-protein kinase C (PKC) signaling mediates the proinflammatory effect of $A_{2A}R$ -mGluR5 interaction in the presence of elevated glutamate levels

We next investigated the signaling pathway associated with the $A_{2A}R$ -mGluR5 interaction-associated proinflammatory effect. As shown in Fig. 7 A, the stimulatory effect of CGS21680 on LPS-induced IL-1 β mRNA expression in mouse neutrophils in the presence of 30 and 300 μ mol/l glutamate was suppressed by the PKC inhibitor GF109203X but not by the PKA inhibitor H-89. Consistent with this, Western blots showed increased PLC expression in the presence of 30 or 300 μ mol/l glutamate, whereas adenylyl cyclase (AC) expression was higher at lower glutamate concentrations (Fig. 7 B). However, in neutrophils from $A_{2A}R$ KO mice or neutrophils

pretreated with mGluR5 siRNA, this glutamate level-dependent change in expression of signaling molecules was not observed (Fig. 7 B). We obtained similar results in human neutrophils: H-89 blocked the antiinflammatory effect of $A_{2A}R$ activation in the presence of 0, 5, and 30 μ mol/l glutamate but had no effect on its proinflammatory effect in the presence of 300 μ mol/l glutamate, which was instead inhibited by GF109203X (Fig. 7 C). The increase in AC expression detected at lower glutamate concentrations was blocked by the $A_{2A}R$ antagonist ZM241385, whereas the significantly elevated PLC levels detected in the presence of high concentrations of glutamate were largely inhibited by a combination of ZM241385 and the mGluR5 antagonist MPEP (Fig. 7 D). These data indicate that the $A_{2A}R$ -mGluR5 complex-dependent proinflammatory effect of $A_{2A}R$ activation that occurs in the presence of elevated glutamate levels is mediated by a PLC-PKC signaling pathway.

The $A_{2A}R$ agonist CGS21680 aggravates and $A_{2A}R$ antagonist ZM241385 attenuates lung damage and neutrophil infiltration in a mouse model of severe TBI-induced ALI

To verify the plasma glutamate-modulated proinflammatory role of BMDC $A_{2A}R$ described above, we investigated the effects of $A_{2A}R$ agonist and antagonist on lung damage in a mouse model of severe TBI-induced ALI. As shown in Fig. 8, in WT mice suffering from severe TBI-induced ALI, the $A_{2A}R$ agonist CGS21680 aggravated lung damage as measured by lung water content (Fig. 8 A), P/F ratio (Fig. 8 B), pulmonary pathological changes (Fig. 8 C), and neutrophil infiltration

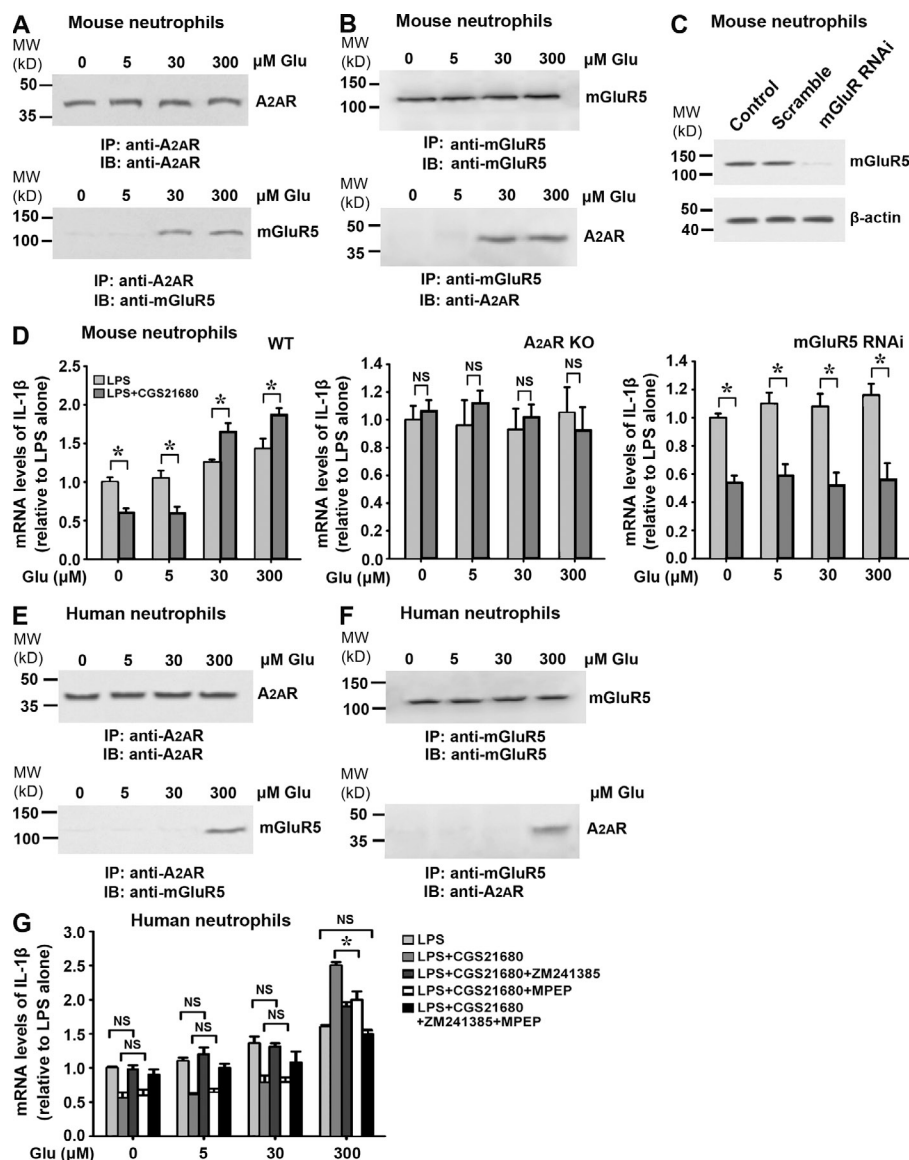


Figure 6. Effect of plasma glutamate levels on $A_{2A}R$ -mGluR5 interaction and associated inflammatory cytokine expressions. (A and B) Co-IP of $A_{2A}R$ and mGluR5 at varying glutamate concentrations in mouse neutrophils using anti- $A_{2A}R$ antibody or anti-mGluR5 as the immunoprecipitating antibody. IB, immunoblot. (C) Western blot for mGluR5 expression in mouse neutrophils to detect the efficiency of mGluR5 RNAi. (D) Real-time PCR for IL-1 β relative mRNA levels in mouse neutrophils. CGS21680: an $A_{2A}R$ agonist; $A_{2A}R$ KO: $A_{2A}R$ gene KO; mGluR5 RNAi: RNA interference of mGluR5 expression. (E and F) Co-IP of $A_{2A}R$ and mGluR5 at varying glutamate concentrations in human neutrophils using anti- $A_{2A}R$ antibody or anti-mGluR5 as the immunoprecipitating antibody. (G) Real-time PCR for IL-1 β relative mRNA levels in human neutrophils. To confirm the effect of $A_{2A}R$ -mGluR5 interaction on IL-1 β expression, the LPS-stimulated human neutrophils were treated with the selective $A_{2A}R$ agonist CGS21680, $A_{2A}R$ antagonist ZM241385, and mGluR5 antagonist MPEP. Data for real-time PCR are expressed as mean \pm SEM. Each experiment was repeated three times. *, $P < 0.01$ between the two groups; NS, no significant difference between the two groups.

into lung tissues (Fig. 8 D). The $A_{2A}R$ antagonist ZM241385 attenuated the lung damage as measured by these parameters (Figs. 8, A–D). These effects of CGS21680 and ZM241385 were not observed in $A_{2A}R$ KO mice (Fig. 8). These data confirm the aggravating effect of $A_{2A}R$ activation in severe TBI-induced ALI and suggest that $A_{2A}R$ antagonist is protective in the treatment of neurogenic ALI.

DISCUSSION

Inflammatory response plays an important role in the progression of ALI. Activation of $A_{2A}R$ is widely considered to be an antiinflammatory event in nonneurogenic ALI, as $A_{2A}R$ inhibits BMDC activation and inflammatory cytokine release. Consistent with this perspective, our findings in an oleic acid-induced ALI model show that global $A_{2A}R$ KO and selective inactivation of BMDC $A_{2A}R$ accelerate lung inflammatory damage. In contrast, in the severe TBI-induced ALI mouse model, selective

inactivation of BMDC $A_{2A}R$ significantly attenuated lung damage and expression of inflammatory cytokines TNF and IL-1 β . This result provides strong evidence that BMDC $A_{2A}R$ activation has opposite effects on nonneurogenic ALI and the neurogenic ALI induced by severe TBI.

Our previous study in mouse models of moderate TBI and in isolated microglia demonstrated that high concentrations of glutamate in the environment can switch the antiinflammatory effect of $A_{2A}R$ activation to a proinflammatory effect (Dai et al., 2010b). This prompted us to measure glutamate levels in blood plasma during BMDC $A_{2A}R$ activation and to investigate whether glutamate levels also play a role in the progression of TBI-induced ALI. Our results show that both in patients with severe TBI-induced ALI and in mouse models of TBI-induced ALI, plasma glutamate levels during the 24 h after injury were much higher than in lung trauma-induced ALI patients or in mice with

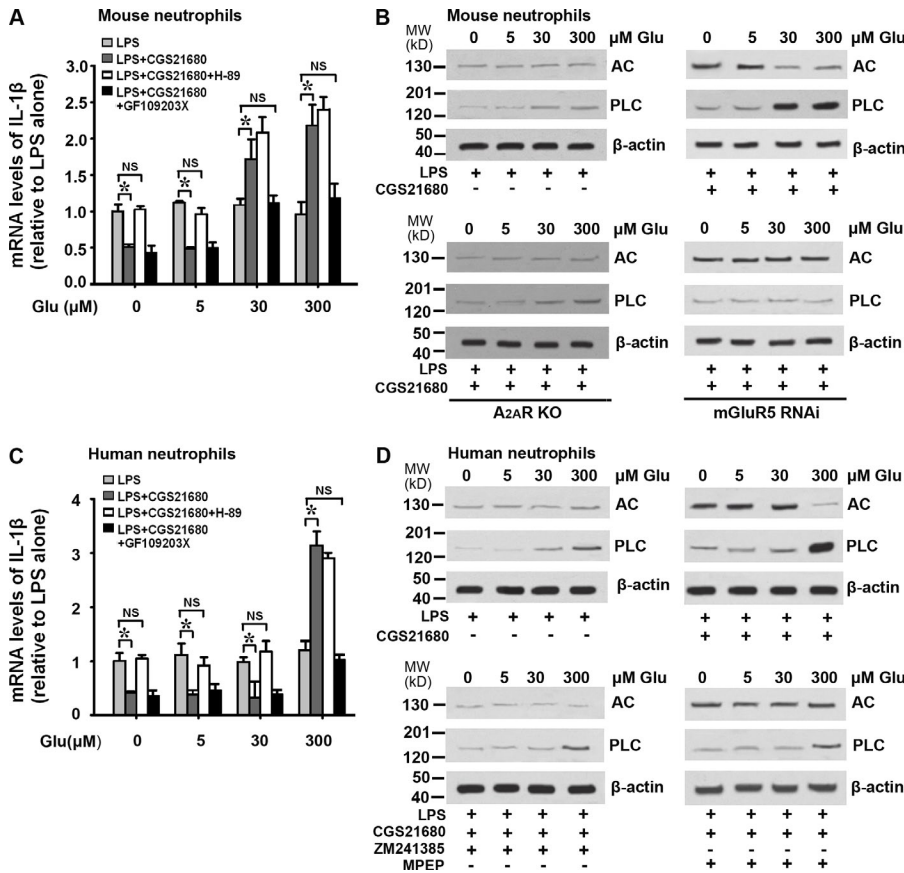


Figure 7. Signaling of the A_{2A}R–mGluR5 interaction-associated pro-inflammatory effect. (A) Real-time PCR for IL-1β relative mRNA levels in mouse neutrophils. CGS21680: an A_{2A}R agonist; H-89: a PKA inhibitor; GF109203X: a PKC inhibitor. (B) Western blot for AC and PLC expression changes in mouse neutrophils. (C) Real-time PCR for IL-1β relative mRNA levels in human neutrophils. (D) Western blot for AC and PLC expression changes in human neutrophils. Data for real-time PCR are reported as mean ± SEM of three independent experiments with three replications in each experiment. *, P < 0.01 between the two groups; NS, no significant difference between the two groups.

oleic acid-induced ALI. Moreover, we found a significant A_{2A}R–mGluR5 interaction in WBCs from patients with severe TBI-induced ALI and WBCs from the mouse model of severe TBI-induced ALI but not in WBCs from patients with trauma-induced ALI or the oleic acid-induced ALI mouse model. This association between plasma glutamate level, lung injury, and BMDC A_{2A}R–mGluR5 interaction was confirmed by the investigation in the mouse model of moderate TBI. In the very small minority of mice suffering from ALI after moderate TBI, we also detected elevated plasma glutamate levels, lung damage, and A_{2A}R–mGluR5 interaction in WBCs. Numerous experimental and clinical studies have demonstrated that CSF or extracellular fluid glutamate concentration increases significantly after TBI, reflecting neuronal and glial glutamate release, diminished glial glutamate uptake, and a damaged blood–brain barrier (Baker et al., 1993; Palmer et al., 1993, 1994; Tomkins et al., 2008). However, further study is required to determine whether the deficiency of glutamate transporter, insufficient capacity of the blood glutamate scavenger oxaloacetate, muscle integrity and muscle breakdown (Lee et al., 1998; O’Kane et al., 1999; Hyde et al., 2003; Hawkins et al., 2006; Zlotnik et al., 2010), or some other mechanism accounts for the increase of plasma glutamate we observed in TBI-induced ALI in the present study.

Previously, functional A_{2A}R–mGluR5 heteromeric complexes had only been reported in the central nervous system

(Ferré et al., 2002; Nishi et al., 2003; Brown et al., 2012). The present study provides the first evidence for this interaction in BMDCs and the first evidence that the complex is induced in ALI. Infiltration of neutrophils associated with inflammatory cytokine release is a hallmark event in the progression of ALI and its severe form, acute respiratory distress syndrome (Baughman et al., 1996; Abraham et al., 2000; Azoulay et al., 2002; Reutershan and Ley, 2004). To confirm that TBI-induced high plasma glutamate levels trigger the A_{2A}R–mGluR5 interaction and that the complex is an important mediator of the proinflammatory effect of BMDC A_{2A}R, we performed additional experiments in isolated human and mouse neutrophils. These in vitro experiments revealed that elevated glutamate levels (300 μmol/l for human neutrophils; 30 and 300 μmol/l for mouse neutrophils) induced the A_{2A}R–mGluR5 interaction. The glutamate levels required to trigger the A_{2A}R–mGluR5 interaction in neutrophils are similar to those detected in patients with TBI-induced ALI and in the mouse model, respectively. In elevated glutamate environments, neutrophil A_{2A}R activation exerted a proinflammatory effect rather than the antiinflammatory effect observed in the presence of lower glutamate concentrations. Moreover, this effect was largely suppressed by either A_{2A}R antagonist ZM241385 or mGluR5 antagonist MPEP and completely blocked by their combination, confirming that A_{2A}R–mGluR5 interaction mediated this proinflammatory effect.

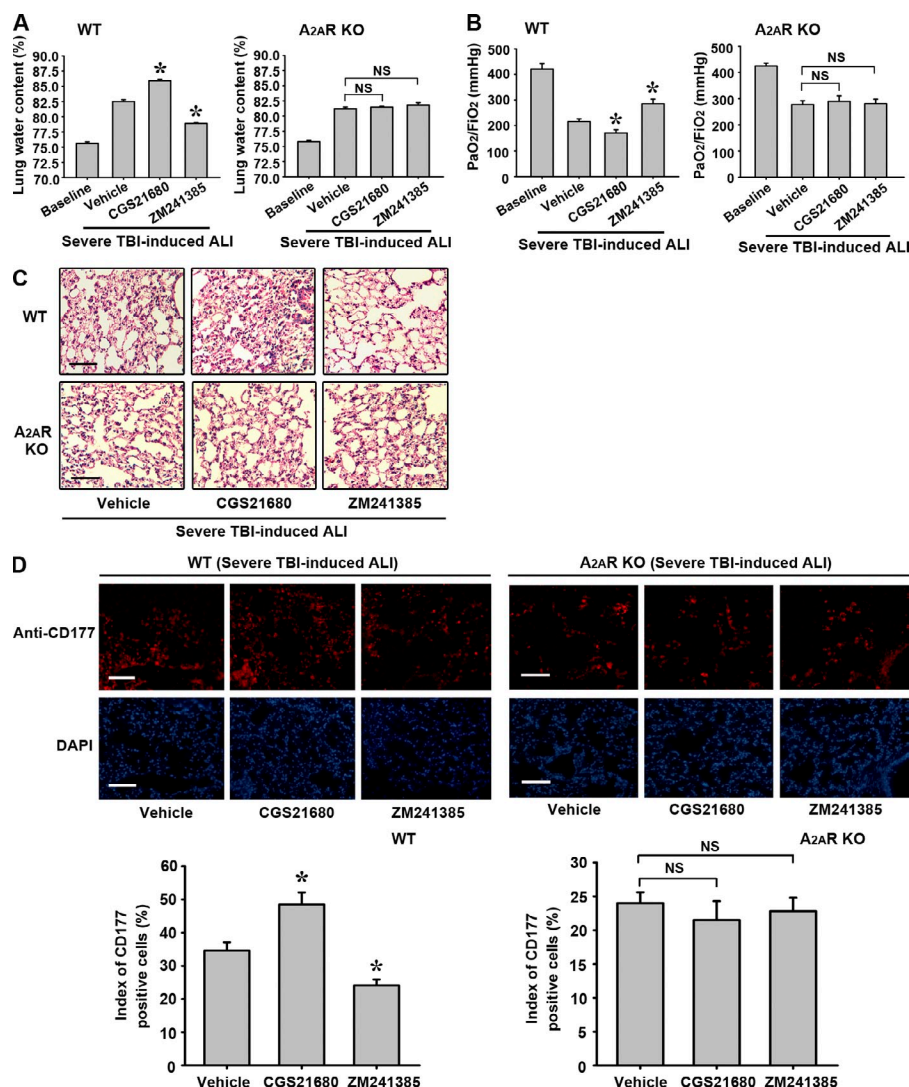


Figure 8. $A_{2A}R$ agonist aggravates, whereas antagonist attenuates lung damage and neutrophil infiltration in the mouse severe TBI-induced ALI model.

Effects of $A_{2A}R$ agonist CGS21680 and antagonist ZM241385 on the treatment of severe TBI-induced ALI. (A) Lung water content. (B) Blood gas analyses of arterial blood. (C) H&E staining for lung sections. (D) Immunofluorescence for neutrophil infiltration with anti-CD-177 antibody; nucleoli were stained by DAPI in WT or $A_{2A}R$ KO mice. Neutrophil counting and statistical analysis were performed by Image-Pro plus version 4.5 and presented as an index of CD-177-positive cells. (A, B, and D) Values are expressed as mean \pm SEM. *, $P < 0.01$ when compared with the vehicle treatment; NS, no significant difference between the two groups. Each experiment was repeated three times. Bars, 100 μ m.

Both $A_{2A}R$ and mGluR5 are G protein-coupled receptors (GPCRs) comprised of a bundle of seven transmembrane (7TM) α -helices connected by three extracellular loops and three intracellular loops (Katritch et al., 2012). Some previous studies have reported that agonists of GPCRs can affect the formation and stability of GPCR homo- or hetero-complexes (Hebert et al., 1996; Angers et al., 2000; Rocheville et al., 2000), provoke changes in downstream signaling, and regulate their functions (Jordan and Devi, 1999; Gomes et al., 2001; Rios et al., 2001; Cheng et al., 2003). These reports provide an explanation for our results. First, $A_{2A}R$ -mGluR5 interaction and this interaction-mediated proinflammatory effect require both $A_{2A}R$ agonist CGS21680 and the presence of a certain concentration of glutamate, the endogenous agonist of mGluR5. Second, this proinflammatory effect is mediated by PLC-PKC signaling rather than by the AC-PKA pathway responsible for the antiinflammatory effect of $A_{2A}R$ activation in low concentrations of glutamate. It is believed that mGluR5 is coupled with Gq, which classically activates the PLC-PKC pathway (Ozawa et al., 1998). $A_{2A}R$ is coupled

with Gs, although AC-cAMP-PKA activation is the predominant downstream signaling pathway, provoking PKA-dependent or -independent PKC signaling (Pinto-Duarte et al., 2005; Fredholm et al., 2007). In the present study, we demonstrate that the PLC-PKC signaling pathway contributes to the glutamate-induced proinflammatory effect of $A_{2A}R$ -mGluR5 interaction in a PKA-independent manner, which accounts for the deleterious effect of BMDC $A_{2A}R$ in severe TBI-induced ALI. Confirming this finding, $A_{2A}R$ antagonist rather than agonist attenuated lung inflammatory damages in severe TBI-induced ALI. Previously, injection of enough glutamate was reported to cause excitotoxic ALI by activating NMDA receptors in lung tissue (Said et al., 1996; Shen et al., 2007), but our results suggest that plasma glutamate-modulated interaction of $A_{2A}R$ and mGluR5 on BMDCs aggravates TBI-induced ALI. It not only presents a novel view of the effect of $A_{2A}R$ in ALI, but also of the role of glutamate in ALI.

In summary, we demonstrate that high levels of glutamate induce the $A_{2A}R$ -mGluR5 interaction in BMDCs to trigger a proinflammatory effect of $A_{2A}R$ activation via PLC-PKC

signaling. This finding offers new insight into the clinical use of $A_{2A}R$ modulators (agonist or antagonist) and glutamate inhibitor for TBI-induced ALI treatment. When plasma glutamate levels are elevated, $A_{2A}R$ antagonist rather than agonist is a potentially beneficial treatment for attenuating lung damage in severe TBI-induced ALI. Importantly, $A_{2A}R$ agonist may actually exacerbate the damage in these conditions.

MATERIALS AND METHODS

Animals. Global $A_{2A}R$ homozygous KO mice and their WT littermates used in this study were developed in the laboratory of J.-F. Chen (Chen et al., 1999) and were generated as previously described (Chen et al., 1999; Dai et al., 2010a,b). In brief, congenic global $A_{2A}R$ KO mice on a C57BL/6 background were generated by backcrossing global $A_{2A}R$ KO on mixed (129-Steel×C57BL/6) genetic background to C57BL/6 mice for 13–15 generations. Heterozygous crossbreeding was used to generate global $A_{2A}R$ KO mice and their WT littermates. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

Patients. ALI is defined as an acute noncardiogenic pulmonary edema with a P/F gradient below 300 mmHg. Hospitalized patients with TBI-induced ALI or lung trauma-induced ALI and their blood cell cultures were eligible for investigation. This study was approved by the Third Military Medical University Human Studies Committee. The clinical information for these patients is presented in [Tables S1 and S2](#).

BMT. To selectively inactivate or reconstitute $A_{2A}R$ on BMDCs, BMT between WT mice and $A_{2A}R$ KO mice was performed as previously described (Yu et al., 2004; Dai et al., 2010a).

Induction of severe TBI-induced ALI and oleic acid-induced ALI. A severe cortical impact was performed by the weight-dropping method (Li et al., 2008, 2009). This reproducible and consistent model is generally associated with 30% mortality within the first 5 min after injury and neurological deficit scores of III within the 24 h after injury (Jin et al., 2008). As in patients, ALI in mice was defined as lung edema with a P/F gradient below 300 mmHg, ruling out heart failure as a mechanism. The nonneurogenic ALI model was induced by intravenous injection of 0.6 ml/kg oleic acid (Sigma-Aldrich). In addition, to confirm the effect of TBI severity on lung damage, a moderate TBI model in mice was also induced via the weight-dropping method. This model yielded a neurological deficit score of II within the 24 h after injury. To investigate the effect of $A_{2A}R$ agonist and antagonist in the mouse severe TBI-induced ALI model, $A_{2A}R$ agonist CGS21680 (0.1 mg/kg) or antagonist ZM241385 (1 mg/kg) was given to the mice at 3 h after TBI.

Assay of lung injury parameters. The lung injury parameters of mice such as lung water content, hematoxylin and eosin (H&E) staining, and TNF and IL-1 β protein levels were assessed as previously described (Liu et al., 2010a). Using goat anti-mouse $A_{2A}R$ antibody (Everest Biotech Ltd.), rabbit anti-mouse CD177 antibody (Beijing Biosynthesis Biotechnology Co., Ltd.), and FITC- or TRITC-conjugated secondary antibody (Beijing Cowin Biotech Co., Ltd.), $A_{2A}R$ location and neutrophil infiltration in lung tissue were determined with standard immunofluorescence immunohistochemistry (Donadieu et al., 2007). Nuclei were then stained by DAPI. The results were analyzed by Image-Pro plus version 4.5 (Media Cybernetics) as described previously (Li et al., 2008). Arterial blood samples were withdrawn into a heparinized syringe by percutaneous left ventricular sampling of lightly anesthetized mice spontaneously breathing room air (Fagan et al., 1999; Xu et al., 2006). Blood gas analysis was immediately performed using the I-STAT Analyzer (Abbott).

Measurement of glutamate level in blood plasma. Glutamate levels in blood plasma of the patients ([Tables S1 and S2](#)) or mice were analyzed by

HPLC using ortho-phthalaldehyde precolumn derivatization and fluorescence detection as previously reported (Aliprandi et al., 2005). Samples were run through a C18 column (Supelcosil) detected by a 157 spectrofluorometric detector (Beckman Coulter) with the excitation wavelength set at 330 nm and the emission wavelength fixed at 450 nm.

WBC isolation and protein extraction. Blood samples from patients or the mouse models were collected in EDTA tubes, and WBCs were prepared by isolating the buffy coat layer after centrifugation at 3,500 rpm for 10 min at 4°C. For the co-IP experiment, WBC proteins were extracted with TRIzol reagent (Invitrogen) from the interphase and lower phenol phase after first removing the upper aqueous phase according to the manufacturer's instructions (Chung et al., 2009).

Pharmacological treatments of isolated neutrophil. Using a discontinuous Percoll gradient (Henson and Oades, 1975; Graham et al., 2007), human neutrophils from healthy donors were isolated from heparinized blood, whereas mouse neutrophils were isolated from BM cells according to the method described previously (Tsai and Collins, 1993; Magalhães et al., 2007). Neutrophils were pretreated with 0, 5, 30, or 300 μ M glutamate for 30 min, followed by a combination of 1,000 ng/ml LPS and 100 nM $A_{2A}R$ agonist CGS21680 for 6 h (Dai et al., 2010b). To elucidate the associated signaling, 1 μ M $A_{2A}R$ selective antagonist ZM241385, 10 μ M PKA inhibitor H-89, 5 μ M PKC inhibitor GF109203X, and mouse mGluR5 siRNA (Santa Cruz Biotechnology, Inc.) and 1 μ M mGluR5 antagonist MPEP (Pacheco et al., 2006) were used. For these cotreatments, CGS21680, ZM241385, and MPEP were added 10 min before LPS treatment. H-89 or GF109203X was added to the cultures 30 min before glutamate was added (Dai et al., 2010b). For the experiments involving mGluR5 siRNA, mouse neutrophils were transfected using the Nucleofection technology (Amaxa) for 24 h: 1.4 μ g of each siRNA was transfected into 1×10^6 cells according to the manufacturer's recommendations. Controls underwent the same transfection procedure. Transfection efficiency was confirmed by Western blot for mGluR5 expression.

Assay of IL-1 β mRNA expressions in neutrophils. 6 h after agents were added, total RNA of neutrophils was isolated using TRIzol reverse transcribed. IL-1 β mRNA expression in neutrophils was evaluated with real-time PCR (Takara Bio Inc.) using the primers described previously (Dai et al., 2010b; Keita et al., 2010). All reported values are presented as mean \pm SEM of three independent experiments conducted in triplicate.

Detection of $A_{2A}R$ and mGluR5 interaction. By using protein A-agarose beads (EMD Millipore) and with the anti- $A_{2A}R$ (Santa Cruz Biotechnology, Inc.), anti-mGluR2/3 (Abcam), or anti-mGluR5 antibodies (Abcam) for human and mouse, co-IP was performed as described previously (Schröder et al., 2009). The immunoreactive bands were developed with the enhanced chemiluminescence detection kit (SuperSignal; Thermo Fisher Scientific).

Western blot for signal molecule expressions. To investigate the associated signaling of $A_{2A}R$ activation, Western blot analysis was also performed using antibodies against AC (Abcam) and PLC (Santa Cruz Biotechnology, Inc.). In these experiments, β -actin (Santa Cruz Biotechnology, Inc.) served as the endogenous control.

Statistical analysis. Statistical comparisons of more than two groups were performed by factorial ANOVA followed by Bonferroni's post hoc test. Graphic data represent mean \pm SEM. A value of $P < 0.01$ was considered statistically significant.

Online supplemental material. Table S1 lists characteristics of patients with severe TBI-induced ALI, including age, initial Glasgow coma scale, and the time of ALI onset. Table S2 lists characteristics of patients with lung trauma-induced ALI. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20122196/DC1>.

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