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# Adenosine $A_{2A}$ Receptor Activation and Macrophage-mediated Experimental Glomerulonephritis

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## Abstract

In immune-induced inflammation, leukocytes are key mediators of tissue damage. Since $A_{2A}$ adenosine receptors ($A_{2A}R$) are endogenous suppressors of inflammation, we examined cellular and molecular mechanisms of kidney damage to determine whether selective activation of $A_{2A}R$ will suppress inflammation in a rat model of glomerulonephritis. Activation of $A_{2A}R$ reduced the degree of kidney injury in both the acute inflammatory phase and the progressive phase of glomerulonephritis. This protection against acute and chronic inflammation was associated with suppression of the glomerular expression of the MDC/CCL22 chemokine and down-regulation of MIP-1α/CCL3, RANTES/CCL5, MIP-1β/CCL4, and MCP-1/CCL2 chemokines. The expression of anti-inflammatory cytokines, IL-4 and IL-10, also increased. The mechanism for these anti-inflammatory responses to the $A_{2A}R$ agonist was suppression of macrophages function. $A_{2A}R$ expression was increased in macrophages, macrophage-derived chemokines were reduced in response to the $A_{2A}R$ agonist, and chemokines not expressed in macrophages did not respond to $A_{2A}R$ activation. Thus, activation of the $A_{2A}R$ on macrophages inhibits immune-associated inflammation. In glomerulonephritis, $A_{2A}R$ activation modulates inflammation and tissue damage even in the progressive phase of glomerulonephritis. Accordingly, pharmacological activation of $A_{2A}R$ could be developed into a novel treatment for glomerulonephritis and other macrophage-related inflammatory diseases.

### Keywords

chemokines; anti-inflammatory cytokines; tissue injury protection

## INTRODUCTION

We still treat immunologically induced inflammatory diseases with nonspecific immunosuppressive drugs that can cause significant morbidity (1). For this reason defining the pathways of organ damage could lead to design of more specific therapies.

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Crescentic glomerulonephritis is a rapidly progressive glomerular disease with a poor prognosis. Macrophages can play an important role in the induction and development of the disease and both the magnitude of proteinuria and the percentage of crescentic glomeruli are correlated with the number of macrophages that infiltrate the glomerulus; this is not the case with the number of $CD8^+$ cells (2,3). Macrophages are constituents of glomerular crescents in progressive crescentic glomerulonephritis and they probably play a major role in the irreversible scarring that leads to kidney failure (4–8). In Wistar-Kyoto rats (WKY), small doses of anti-glomerular basement membrane (GBM) antibody induce proliferative and necrotizing glomerulonephritis with crescent formation plus infiltration of $CD8^+$ cells and macrophages into the glomeruli (9–13). In this model macrophages accumulate in glomeruli during later phases and contribute to the progressive decline in kidney function (3,14).

Chemokines mediate selective attraction and activation of various subsets of leukocytes (6, 15–17). In the induction of anti-GBM glomerulonephritis in WKY rats CC chemokines play an important role. We found that CC chemokines, MCP-1/CCL2, MIP-1β/CCL4, RANTES/CCL5, and MDC/CCL22, and the CX3C chemokine, fractalkines/$CX_3CL1$, are induced during the disease and that the expression of multiple chemokines coincides with the influx of $CD8^+$ cell and $ED1^+$ macrophages into the glomeruli. In *in vitro* studies these chemokines induced strong migratory response in inflammatory cells prepared from the nephritic glomeruli (9–11).

The degree of inflammation can be tightly regulated by endogenous anti-inflammation pathways, including, PPARγ, adiponectin, NO, and adenosine receptors (18–24). Genetic and pharmacological evidence support a non-redundant role for both endogenous adenosine and $A_{2A}$ receptors in protecting against acute inflammatory damage in models of inflammatory injury and systemic inflammation (25). The anti-inflammatory mechanism involves downregulation of activated immune cells *in vivo* (25). Thus, the $A_{2A}R$ could modulate inflammatory processes because it is expressed on most cells involved in inflammation, including a variety of hematopoietic cells, endothelial cells, and smooth muscle cells (19,20, 25,26). We have found that quiescent peritoneal macrophages do not express $A_{2A}R$ but following addition of LPS, these cells express abundant $A_{2A}R$. *In vitro* studies indicate that $A_{2A}R$ are present on polymorphonuclear leukocytes (PMN) and can inhibit the expression of β2-integrins and adhesion, can suppress oxygen radical production, degranulation, and production of TNF-α (27–31). In addition, expression and activation of $A_{2A}$ receptor on macrophages can inhibit the production of IL-12, TNF-α, and nitric oxide, and enhance the secretion of IL-10 in response to lipopolysaccharide (LPS) (32–35). Adenosine can inhibit proliferation, activation, and production of inflammatory cytokines in peripheral T-cells it also can enhance the production of anti-inflammatory cytokines (36,37).

Because of the potential for beneficial effects of pharmacological activation of $A_{2A}R$ as a therapeutic target, we tested the hypothesis that $A_{2A}R$ activation would protect against immune-mediated inflammation in experimental glomerulonephritis. Activation of $A_{2A}R$ significantly reduced leukocyte infiltration into the kidney and prevented kidney injury during the acute inflammatory phase and the progressive phase of glomerulonpehritis. Our results indicate that activation of $A_{2A}R$ represents a potential therapeutic strategy for glomerulonephritis and possibly other macrophage-mediated diseases.

## MATERIAL AND METHODS

### Induction, treatment, and analysis of anti-GBM glomerulonephritis

Animal studies were approved by the IACUC at Baylor College of Medicine. Male WKY rats (Harlan Sprague Dawley Inc., Indianapolis, IN), weighing 180–200 grams received an intravenous injection of 25μl/100g body weight of anti-GBM Ab as described (9,10,12,38).

We activated $A_{2A}R$ with a selective $A_{2A}$ receptor agonist, CGS 21680, (Tocris Cookson Inc., Ellisville, MO, 1.5mg/Kg i.p. twice a day, n = 6) and inactivated $A_{2A}R$ using a selective receptor antagonist, ZM241385 (Tocris, 2mg/Kg i.p. twice daily, n = 6) for a period of 5 days, as described (25). In initial experiments, we pharmacologically activated or inhibited $A_{2A}R$ during the acute inflammatory phase of the disease by starting treatment 8 hours after the injection of anti-GBM antibody (maximum glomerular deposition of IgG occurs one hour after injection of anti-GBM antibody (39)). In the second set of experiments we began treatment with $A_{2A}R$ agonist at day six after the injecting the anti-GBM antibody (the progressive phase of crescentic glomerulonephritis). Urine protein was assayed using sulfosalicylic acid and rats were euthanized at day six, or day 12 to collect blood and kidney tissues. During the first hour after injecting the $A_{2A}R$ agonist or the $A_{2A}R$ antagonist, tail-cuff blood pressure was measured in conscious rats (Visitech Systems, Apex NC) as described (40).

### mRNA expression of $A_{2A}$ R, chemokines, cytokines, and adhesion molecules

Rat $A_{2A}R$ nucleotides 459 to 801 (GenBank sequence accession number NM_053294) were used to generate $A_{2A}R$ probe from brain tissue by RT-PCR. MDC/CCL22 (400 bp), RANTES/CCL5 (246 bp), MIP-1a/CCL3 (284 bp), factalkine/CX3CL1 (420 bp), MIP-3β/CCL19 (380 bp), MIP-1β/CCL4 (210 bp), MCP-1/CCL2 (239 bp), VCAM-1 (371 bp), ICAM-1 (292 bp), and L-32 (92 bp) riboprobes were generated by PCR reaction using cDNA templates. rCK1 (BD Pharmingen, San Diego, CA) was used to investigate cytokine expression. Glomeruli were prepared by sequential sieving and total RNA was isolated from glomeruli (11,41). Three μg of total RNA from each sample was used in an RNase protection assay using the Torrey Pines Biolabs kit (Houston, TX) as described (11,16,42,43). Phosphoimage quantitiation was performed using the PhosphorImager SI scanning instrument and ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA) (16,44,45).

### Morphological analysis, immunohistochemical phenotyping, and quantitation of leukocytes

Kidney samples fixed in formalin or methanol-Carnoy fixative solution were embedded in paraffin. Two to three-μm sections were stained with periodic acid-Schiff reagent to assess glomerular hypercellularity, necrotizing lesions, and formation of glomerular crescents (crescentic glomeruli per 100 glomeruli was calculated and expressed as a percentage). Infiltrating leukocytes were immunohistochemically stained for $CD8^+$ and $ED1^+$, as described (9,11,12). Positively stained cells per 100 glomeruli were counted and expressed per glomerular section. All quantitative morphological analyses were performed in a blinded fashion.

### Immunohistochemistry of $A_{2A}R$, $ED1^+$ cells, $CD8^+$ cells, mesangial cells, and podocytes

Paraffin sections of methanol-Carnoy fixed tissue were stained with goat polyclonal anti-$A_{2A}R$ antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibody ED-1 against rat macrophages (Chemicon, Temecula, CA), or monoclonal OX-8 against rat CD8 (BD Biosciences Pharmingen). Two-color dual antigen immunostaining was obtained by serial avidin-biotin peroxidase and alkaline phosphatase staining reactions with final chromogenic substrates of diamibenzidine (brown color) and Fast Red (red color), respectively as described (12,46,47).

Kidney sections were also stained with α-SMA (Dako, Carpinteria, CA), a marker of activated mesangial cells or α-Actinin-4 (AXXORA, LLC, San Diego, CA), a marker for podocytes (48,49).

# RESULTS

## $A_{2A}$ R expression in nephritic glomeruli

Normal glomeruli do not express $A_{2A}$ R. In response to the anti-GBM antibody, however, there was strong expression of $A_{2A}$R from day 3 onwards (Figure 1 A). The glomerular level of $A_{2A}$R mRNA was 6.2 fold higher in anti-GBM Ab-treated rats than in normal glomeruli on day 3. $A_{2A}$R expression peaked at day 5 (6.8-fold increase) and started to decrease by day 7 (5.2-fold increase). Double immunohistochemical staining of $ED1^+$ cells and $A_{2A}$R demonstrated that $A_{2A}$R was virtually confined to macrophages. There was some staining in the location of glomerular endothelial cells (Figure 1 B) but $A_{2A}$R was not expressed in either $CD8^+$ cells (Figure 1 C) or in monocytes located in extraglomerular vessels (Figure 1 D). These results suggest that increased expression of $A_{2A}$R in macrophages might yield an anti-inflammatory defense against kidney damage.

## $A_{2A}$R activation prevents glomerulonephritis

In the acute (day 1 to day 5) phase of crescentic glomerulonephritis control rats exhibited severe glomerular hypercellularity, necrotizing lesions, and crescentic formation (Figure 2 and Figure 3). Increased expression of α-SMA, a marker for mesangial cells injury, was inversely correlated with reduced α-Actinin-4, a marker for podocytes, indicating the degree of glomerular injury (Figure 4). In rats treated with CGS21680, a specific $A_{2A}$R agonist, however, there was only minimal damage to the kidneys. Notably, crescent formation, a major characteristic of this model, was abolished by CGS21680 treatment and necrotizing lesions were not observed while glomerular hypercellularity was markedly attenuated by ~ 70% (Figure 2 and Figure 3 a). Treatment with the $A_{2A}$R agonist also decreased α-SMA expression and restored the expression of α-Actinin- 4 (Figure 4). There also was no proteinuria (Figure 5 a). In contrast, in rats with anti-GBM glomerulonephritis treated with a specific antagonist for $A_{2A}$R, ZM241385, there was more severe glomerular hypercellularity, necrotizing lesions, and crescentic formation compared to control rats (Figure 2 and Figure 3 a). $A_{2A}$R antagonist also enhanced the expression of α-SMA and increased the loss of α-Actinin- 4 (Figure 4). Finally, proteinuria was also significantly higher in ZM241385-treated vs. control rats with glomerulonephritis (Figure 5 a). Thus, selective activation of $A_{2A}$R at acute phase (day 1 to day 5) confers kidney protection from damage.

Increased or normal blood pressure has been reported in mice lacking $A_{2A}$R. We determined if $A_{2A}$R antagonism could increase blood pressure and contribute to kidney damage (46,50). There was no significant difference in systolic blood pressure among the groups at day 4 or day 6 after induction of anti-GBM GN (D4: 147.75 ± 18.7, 141.0 ± 14.2, 149.7 ± 14.1; D6: 134.6 ± 8.01, 131.0 ± 9.43, 137.0 ± 5.6 in control, $A_{2A}$R agonist, and$A_{2A}$R antagonist treated rats respectively).

Next, we treated rats in the progressive phase of glomerulonephritis (day 6 to day 11) using the $A_{2A}$R agonist, CGS21680. As shown in Figure 2 and Figure 3b, glomerular hypercellularity, necrotizing lesions, and crescentic formation were markedly reduced at day 12 compared to the results in the control group (88%, 90%, and 74%, respectively). $A_{2A}$R activation also decreased α-SMA expression and restored the expression of α-Actinin-4 vs. results in the control group (Figure 4). Finally, CGS21680 reduced proteinuria within 24 hours of administration; the inhibitory effect was sustained during the treatment period (Figure 5 b). Thus, selective activation of $A_{2A}$R also reduces kidney injury and improves proteinuria during the progressive phase of glomerulonephritis.

### Prevention of glomerulonephritis by $A_{2A}R$ activation reduces lymphocyte/macrophage infiltration

To understand the mechanism for the prevention of kidney-injury, we examined the infiltration of leukocytes into the kidney. As shown in Figure 2 and Figure 3a there was a prominent accumulation of $CD8^+$cells and $ED1^+$ macrophages in glomeruli of control rats during the acute (day 1 to 5) inflammatory phase of glomerulonephritis. Treatment with CGS21680 dramatically attenuated infiltration of $CD8^+$ cells and $ED1^+$ macrophages into glomeruli. Pharmacological activation of $A_{2A}R$ in the progressive phase (from day 6 to day 11) blocked macrophage infiltration to levels only 26% of the level in glomeruli of control rats (Figure 2 and Figure 3 b). This model of glomerulonephritis is characterized by an early infiltration of $CD8^+$ cells with a maximum increase on day 3 after the injection of anti-GBM Ab (13). Consequently, $CD8^+$ cells were not observed in either control or CGS21680 treated rats.

### $A_{2A}$ R activation modulates the expression of chemokines and cytokines in anti-GBM glomerulonephritis

A potential mechanism by which activation of $A_{2A}R$ protects from inflammatory damage and leukocyte infiltration could be via suppression of cytokines/chemokines. We found that pharmacological $A_{2A}R$ activation in the acute inflammatory phase (day 1 to day 5) did not reduce glomerular expression of pro-inflammatory cytokines IL-1β or TNF-α mRNA. In contrast, it significantly increased the expression of anti-inflammatory cytokines, IL-4 and IL-10 (69% and 66% respectively), compared to levels in glomeruli of control rats (Figure 6 a and Figure 7a). Activation of $A_{2A}R$ abolished the induction of MDC/CCL22 and significantly attenuated the expression of MIP-1a/CCL3, MIP-1β/CCL4, RANTES/CCL5, and MCP-1/CCL2 (Figure 6 b, c and 7 b, c) but did not affect expression of fractalkine/$CX_3CL1$ (Figure 6 c and Figure 7 c). Interestingly, inhibition of $A_{2A}R$ by ZM241385 did not significantly change the expression of these chemokines. Expression of anti-inflammatory cytokines seemed to be attenuated by ZM241385 but the changes were not statistically significant compared to the control results. When compared with levels in CGS21680-treated rats, IL-10 and IL-4 were significantly reduced in rats treated with ZM241385 (64.2% and 61% respectively) (Figure 6 a and Figure 7 a).

We also investigated the effects of the $A_{2A}R$ agonist and antagonist on the expression of VCAM-I and ICAM-1 in anti-GBM glomerulonephritis. Increased expression of VCAM-1 and ICAM-1 was observed in anti-GBM glomerulonephritis and $A_{2A}R$ activation or inactivation did not significantly modify their expression in the acute inflammatory phase (day 1 to day 5) compared to control rats (Figure 8).

Pharmacological activation of $A_{2A}R$ in rats with established glomerulonephritis (day 6 to day 11), significantly enhanced the expression of the anti-inflammatory cytokine IL-4 (104%) but not of IL-10 (Figure 6 d and Figure 7 d). The induction of MDC/CCL22 chemokine was suppressed, but the expression of other chemokines and VCAM-1 or ICAM-1 did not change. Fractalkine/$CX_3CL1$ expression significantly increased (Figure 6 e, f;Figure 7 e, f, and Figure 8).

These results suggest that $A_{2A}R$ pharmacological activation modulates renal injury by altering expression of inflammatory and anti-inflammatory cytokines in the kidney.

## DISCUSSION

Our results indicate that activation of adenosine $A_{2A}$ receptors prevents infiltration of leukocytes into the kidney, suppresses glomerular inflammation, and protects the kidney from inflammatory injuries induced by anti-GBM glomerulonephritis. These benefits were observed

both in the acute inflammatory phase and during the progressive phase of glomerulonephritis. The protection can be linked to the ability of activated $A_{2A}R$ to suppress infiltration of leukocytes, the key mediators of kidney damage.

We found that $A_{2A}R$ activation alters the expression of chemokines. Specifically, pharmacological activation of $A_{2A}R$ suppresses MDC/CCL22 expression and markedly reduces the expression of other chemokines during the acute inflammatory phase of glomerulonephritis (Figure 6). These changes in chemokine expression can explain the decreased infiltration of $CD8^+$ and macrophages (6,9–11). During the progressive phase of glomerulonephritis (day 6 to day 11), we found suppressed MDC/CCL22 induction while the expression of other chemokines (except for fractalkine/$CX_3CL1$) was not modified. These results are consistent with the report that MDC/CCL22 plays a critical role in stimulating the influx of macrophages into glomeruli in anti-GBM glomerulonephritis (11). Notably, MDC/CCL22 expression in anti-GBM glomerulonephritis is confined to macrophages (11). As shown in Figure 1, at day 7 after injecting the anti-GBM antibody, macrophages are the major cellular site of $A_{2A}R$ expression in nephritic glomeruli. When MDC/CCL22 expression is inhibited by the $A_{2A}R$ agonist, CGS21680, there also is a dramatic inhibition of $ED1^+$ macrophage infiltration. These results raise the following possibilities: 1) Activation of macrophage-specific $A_{2A}R$ suppresses MDC/CCL22 expression resulting in decreased macrophage infiltration in the acute phase of glomerulonephritis; and 2) macrophage infiltration in later stages of glomerulonephritis depends on MDC/CCL22 because blocking expression of this chemokine ameliorates the degree of functional damage. Interestingly, $A_{2A}R$ activation in the progressive phase of glomerulonephritis did not alter the expression of other chemokines, except for fractalkine/$CX_3CL1$. Possibly increased fractalkine/$CX_3CL1$ expression occurs because of endothelial cell activation in the nephritic glomeruli. This interpretation is suggested because fractalkine/$CX_3CL1$ is mainly expressed by endothelial cells and we have shown that fractalkine/$CX_3CL1$ is induced in the endothelium of nephritic glomeruli (10). In contrast, we have not found $A_{2A}R$ in cultured glomerular endothelial cells (quiescent or activated) or HUVEC (quiescent). Thus, a plausible explanation for the finding that $A_{2A}R$ occupancy during the progressive phase of glomerulonephritis does not alter the expression of other chemokines, is that the chemokines at day 6 to day11 could be produced by $A_{2A}R$-negative kidney cells.

Our finding that activation of $A_{2A}R$ did not suppress the expression of pro-inflammatory cytokines, IL-1β and TNF-α, (Figure 5 a and d) is consistent with the notion that macrophages are not the predominant source of IL-1β and TNF-α in this model of glomerulonephritis (51, 52). On the other hand, *in vitro* studies indicate that IL-1β and TNF-α can up-regulate $A_{2A}R$ in THP-1 cells, human dermal microvascular endothelial cells, and PC12 cells (35, 53, 54). Our results, therefore, suggest that IL-1β and TNF-α could induce $A_{2A}R$ expression in other cells and contribute to the beneficial effect of $A_{2A}R$ agonists in preventing glomerular damage.

In addition, we found that the expression of the anti-inflammatory cytokines, IL-10 and/or IL-4, was enhanced by the $A_{2A}R$ activation during anti-GBM glomerulonephritis. These cytokines might also contribute to the beneficial effects of $A_{2A}R$ activation on kidney injury (55,56).

Increasing evidence suggest that $A_{2A}R$ tissue-protection requires activation of receptors expressed on bone marrow-derived cells (57,58). Our data indicate that macrophages are the target cells mediating the benefits of $A_{2A}R$ activation in both the acute and progressive phases of glomerulonephritis. First, $A_{2A}R$ expression was increased in macrophages but not in $CD8^+$ cells during anti-GBM GN (Figure 1B and 1C). Second, macrophage derived-chemokines such as MDC/CCL22, MCP-1/CCL2, RANTES/CCL5, and MIP-1α/CCL3 were decreased in response to $A_{2A}R$ agonist. Third, chemokines not expressed in macrophages (e.g.,

fractalkine/CX3CL1) were unaffected by the $A_{2A}R$ agonist. Finally, macrophages are present in the crescents and in progressive glomerulonephritis and are correlated with the degree of glomerular injury (5,6,8). It has been found that depletion of macrophages in this and other models of glomerulonephritis suppresses glomerular crescent formation and reduces proteinuria (3,7,59). Accordingly, suppression of the expression of MDC/CCL22 and reduced expression of other chemokines when macrophage-$A_{2A}R$ activation occurs could explain in part the beneficial effect of CGS21680 in both acute and progressive phases of anti-GBM glomerulonephritis.

Notably, we found that the $A_{2A}R$ agonist also was beneficial in the late phase of anti-GBM glomerulonephritis when macrophage influx and crescent formation were at their maximum (day 6) (13,60). These responses were associated with a significant reduction in macrophage infiltration (74%) suggesting that activation of $A_{2A}R$ on macrophages was responsible for the suppression of glomerular injury.

To examine mechanisms underlying the beneficial effects of $A_{2A}R$ we determined the expression of α-SMA, a marker for mesangial cell injury and activation (49)and α-Actinin-4, a marker for podocytes, in glomeruli of rats with anti-GBM glomerulonephritis. Earlier reports have demonstrated that changes in podocytes contribute to the severity of anti-GBM glomerulonephritis (48,61,62). We found that the expression of α-SMA is increased and that α-Actinin- 4 expression is reduced in anti-GBM GN. Treatment with $A_{2A}R$ agonist significantly decreased α-SMA expression and restored the expression of α-Actinin- 4. In contrast, $A_{2A}R$ antagonist enhanced the expression of α-SMA and increased the loss of the podocyte marker, α-Actinin- 4, compared to the controls rats. These data suggest that $A_{2A}R$ agonist may attenuate kidney injury through effects on mesangial cells and podocytes. Future experiments will determine if $A_{2A}R$ activation prevents mesangial cells injury and restores α-Actinin-4 by reducing inflammation or through a direct effect on mesangial cells and podocytes.

$A_{2A}R$ activation or inactivation did not modify blood pressure in rats with anti-GBM glomerulonephritis. Earlier reposts in mice lacking $A_{2A}R$ provide inconsistent changes in blood pressure (46,50). The inconsistent results could reflect differences in strains of mice, administration of $A_{2A}R$ agonists, conscious vs. anesthetized animals, etc. Regardless, the effects of the $A_{2A}R$ agonist and antagonist we found are not related to changes in blood pressure.

Prolonged exposure to adenosine agonists could produce **d**esensitization of $A_{2A}R$ (63–65). In certain reports, desensitization was dose-dependent and reversible; suggested mechanisms for desensitization have included down-regulation of the expression of $A_{2A}R$ or of Gs proteins involved in the cellular signaling or phosphorylation of the $A_{2A}$, G protein-coupled receptor (GPCR) by GPCR kinases. There are also reports, however, providing evidence that prolonged stimulation of $A_{2A}R$ does not lead to loss of functional response to adenosine agonists (66, 67). In our experiments, treatment with adenosine agonists increased expression of $A_{2A}R$ in glomeruli of rats with anti-GBM glomerulonephritis, providing a mechanism that would prevent desensitization (Supplementary results). Interestingly, in rats treated with ZM241385, we found decreased expression of $A_{2A}R$. This finding could eliminate responses to endogenous adenosine-induced activation of $A_{2A}R$ and lead to additional kidney damage.

In summary, we have demonstrated that pharmacological activation of $A_{2A}R$ attenuates leukocyte infiltration into the kidney during both the acute and progressive phases of anti-GBM glomerulonephritis. Subsequent responses include decreased expression of chemokines and upregulation of anti-inflammatory cytokines with suppressed inflammation. These events are associated with marked protection against glomerular injury. Consequently, $A_{2A}R$ activation

could be a novel therapeutic strategy for modifying macrophage-mediated glomerulonephritis or other diseases dependent on macrophages.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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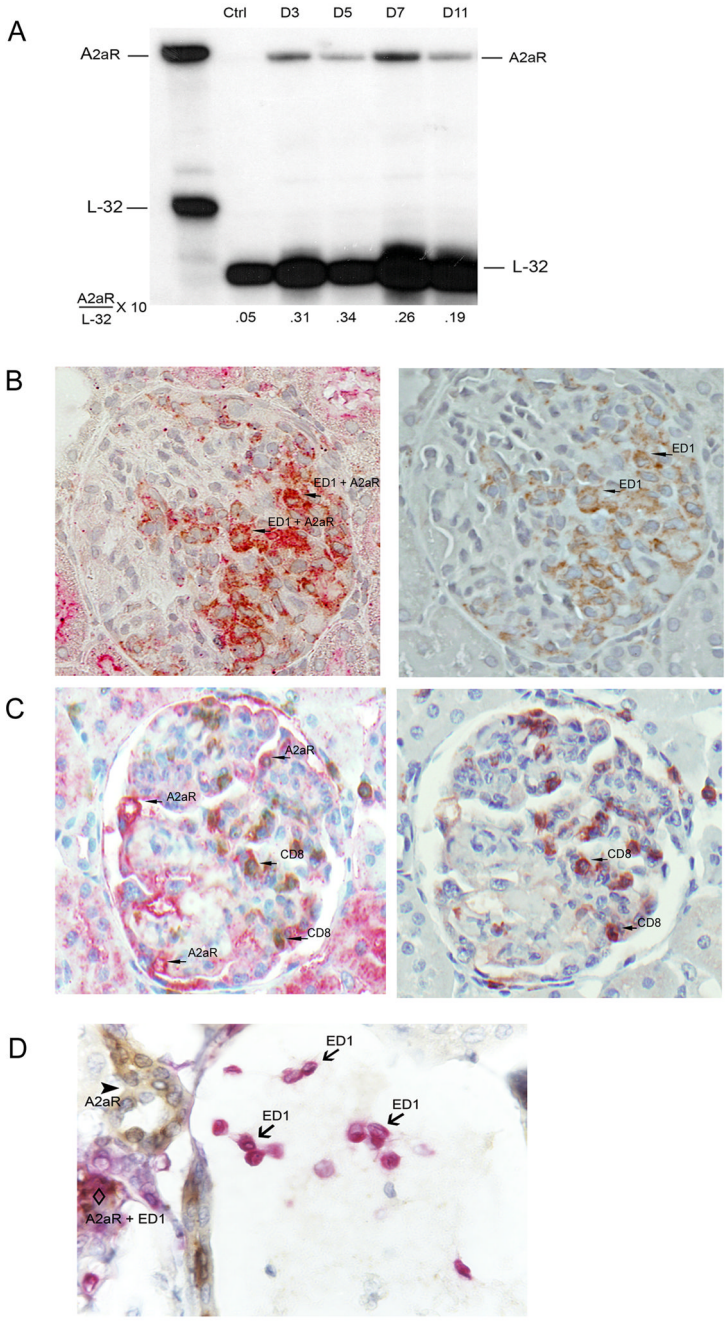
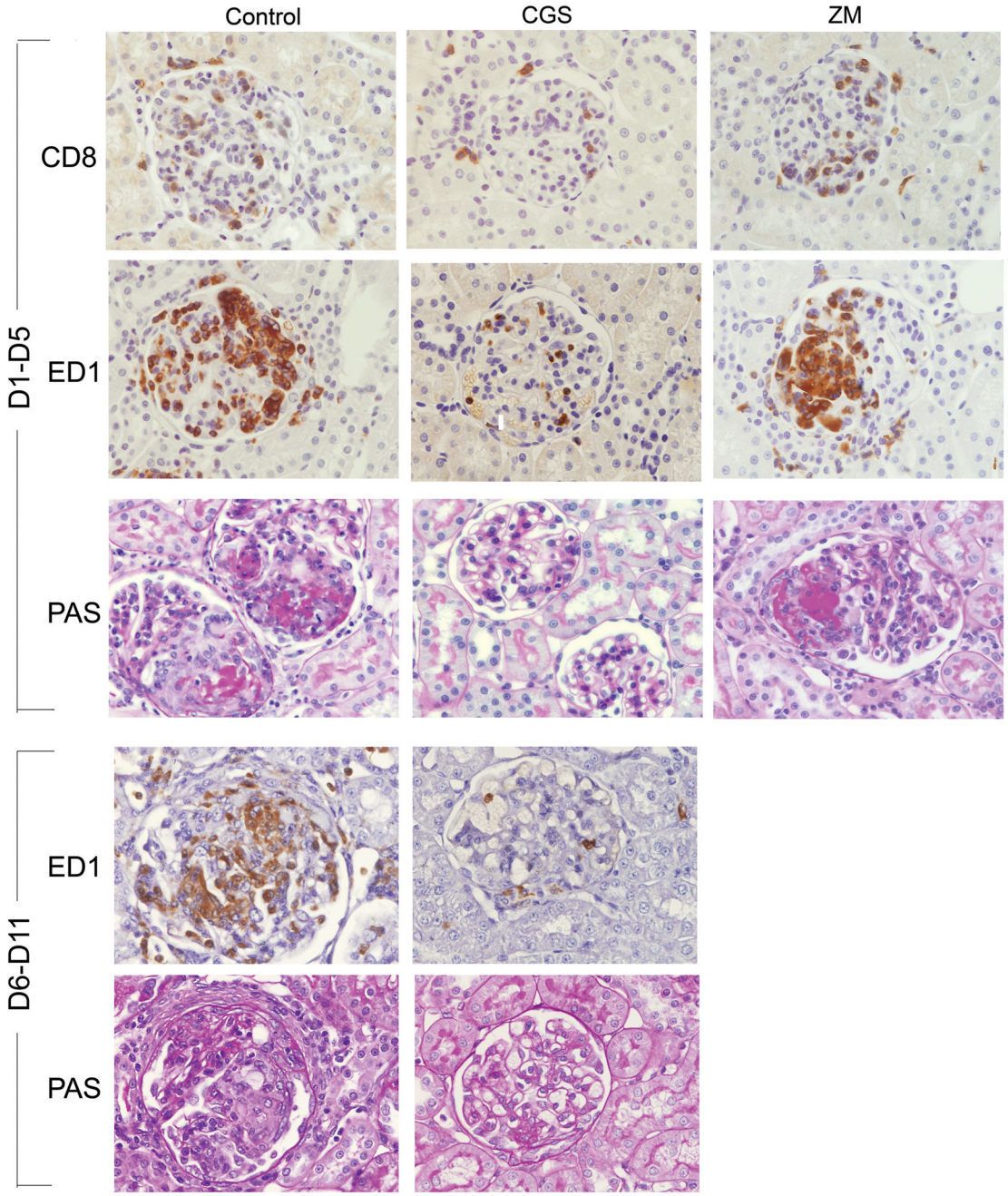



**1.**

Induction of $A_{2A}R$ in nephritic glomeruli. (A) RNase protection analysis was performed to determine $A_{2A}R$ mRNA expression in the glomeruli of anti-GBM glomerulonephritis in WKY rats. $A_{2A}R$ was induced from day 3 onwards during anti-GBM glomerulonephritis. The expression of $A_{2A}R$ peaked at day 5 and started to decrease by day 7. Rat ribosomal L-32 gene was used as a housekeeping gene. Probe contains polylinker regions and is longer than the protected bands. The expression levels of $A_{2A}R$ mRNA are expressed relative to the mRNA levels of the housekeeping gene L-32. Data shown is a representative of three separate experiments. (B) Double immunohistochemical staining of $ED1^+$ cells (brown color) overlap

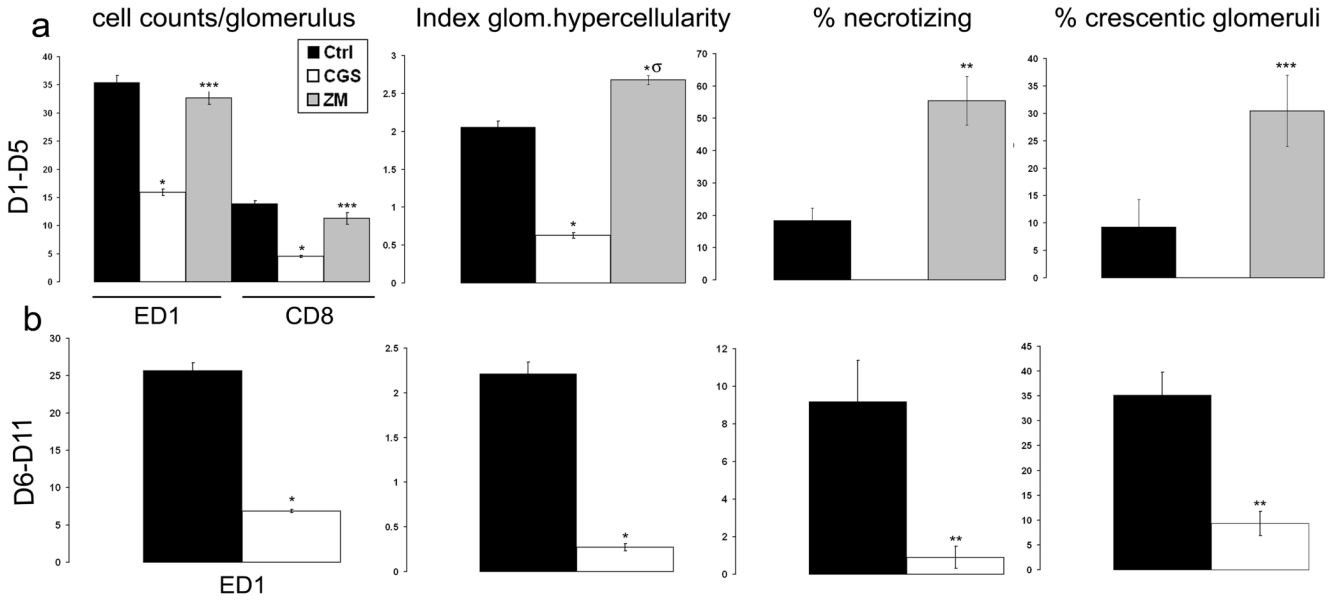
with $A_{2A}R$ (red color), thus $A_{2A}R$ was mainly in Mϕ. (C) Interestingly, $A_{2A}R$ (red color) was not expressed in CD8+ cells (brown color) or (D) monocytes (red color) located in extraglomerular vessels. (B) and (C), right panel, show $ED1^+$ or $CD8^+$ cells staining after removal of red color ($A_{2A}R$ staining) from (B) and (C) slides at left panel to demonstrate the overlap of $A_{2A}R$ expression in ED1+ but not in CD8+ cells. Sections for staining were sampled on day 7 after anti-GBM antibody injection.




**2.**

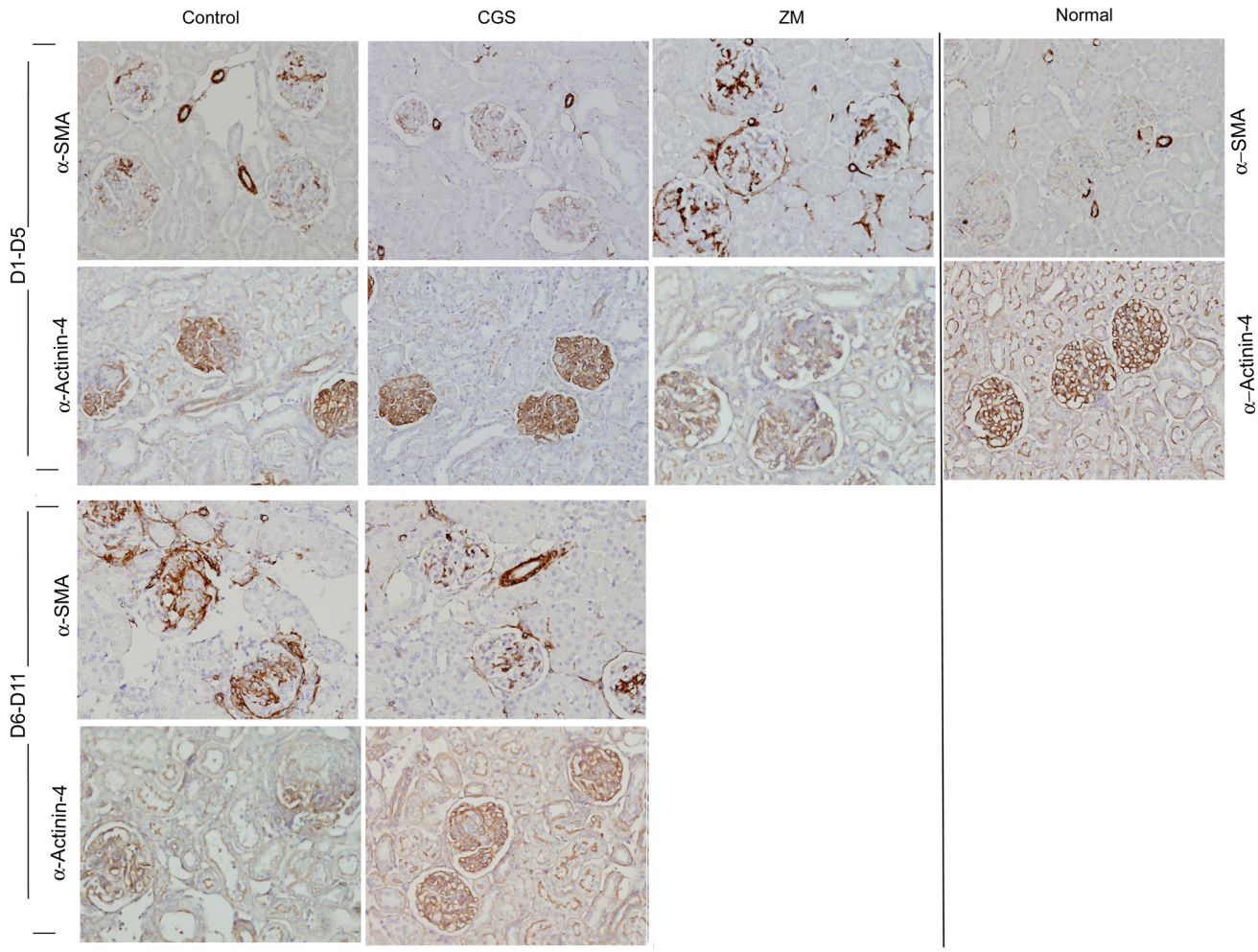
$A_{2A}R$ attenuates inflammatory infiltrates and renal injury during the acute inflammatory phase and progressive phase in anti-GBM glomerulonephritis. Immunohistochemistry stained for $CD8^+$ cells or $ED1^+$ monocytes/macrophages, and periodic acid-Schiff staining of kidney sections of control, CGS216809 (CGS), and ZM241385 (ZM)-treated rats with anti-GBM glomerulonephritis. In the acute phase of the disease (day 1 to day 5), the control group of glomerulonephritis rats display severe glomerular hypercellularity, necrotizing lesions, crescentic formation, and prominent accumulation of $CD8^+$ and $ED1^+$ macrophages infiltration. In CGS-treated rats, crescentic formation and necrotizing lesions were prevented, and $CD8^+$ and $ED1^+$ macrophages infiltrates attenuated. Worsening of glomerular injury was

observed in rats treated with ZM. In the progressive phase of the disease (day 6 to day 11) CGS markedly reduced glomerular lesion and $ED1^{+}$ cell infiltrate.



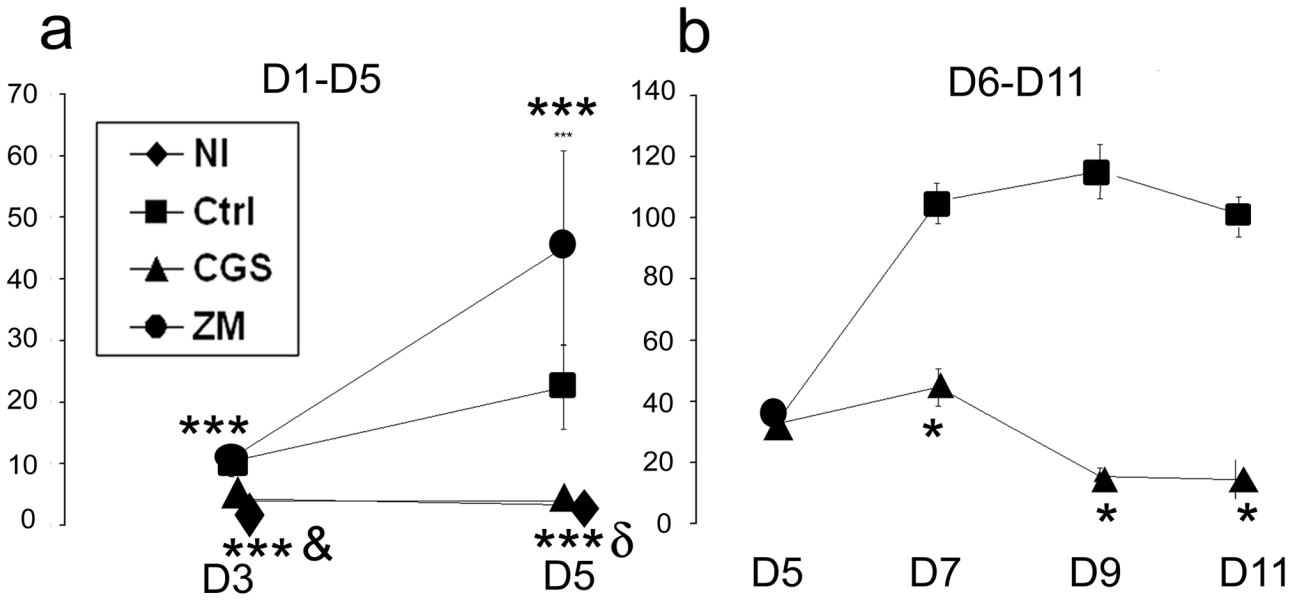

**3.**

Quantitation of $CD8^+$ and $ED1^+$ cell infiltration, glomerular hypercellularity, necrotizing lesions, and crescent formation in the glomeruli from WKY rats with anti-GBM glomerulonephritis that were treated with vehicle, CGS21680 (CGS) or ZM241385 (ZM) during day 1 to day 5 and day 6 to day 11. One hundred glomeruli per section were counted. Each data point represents sections sampled from six rats and is expressed as mean ± SEM. *p<0.0001 vs. control, **p<0.005 vs. control, ***p<0.05 vs. control, σp<0.0001 vs. CGS



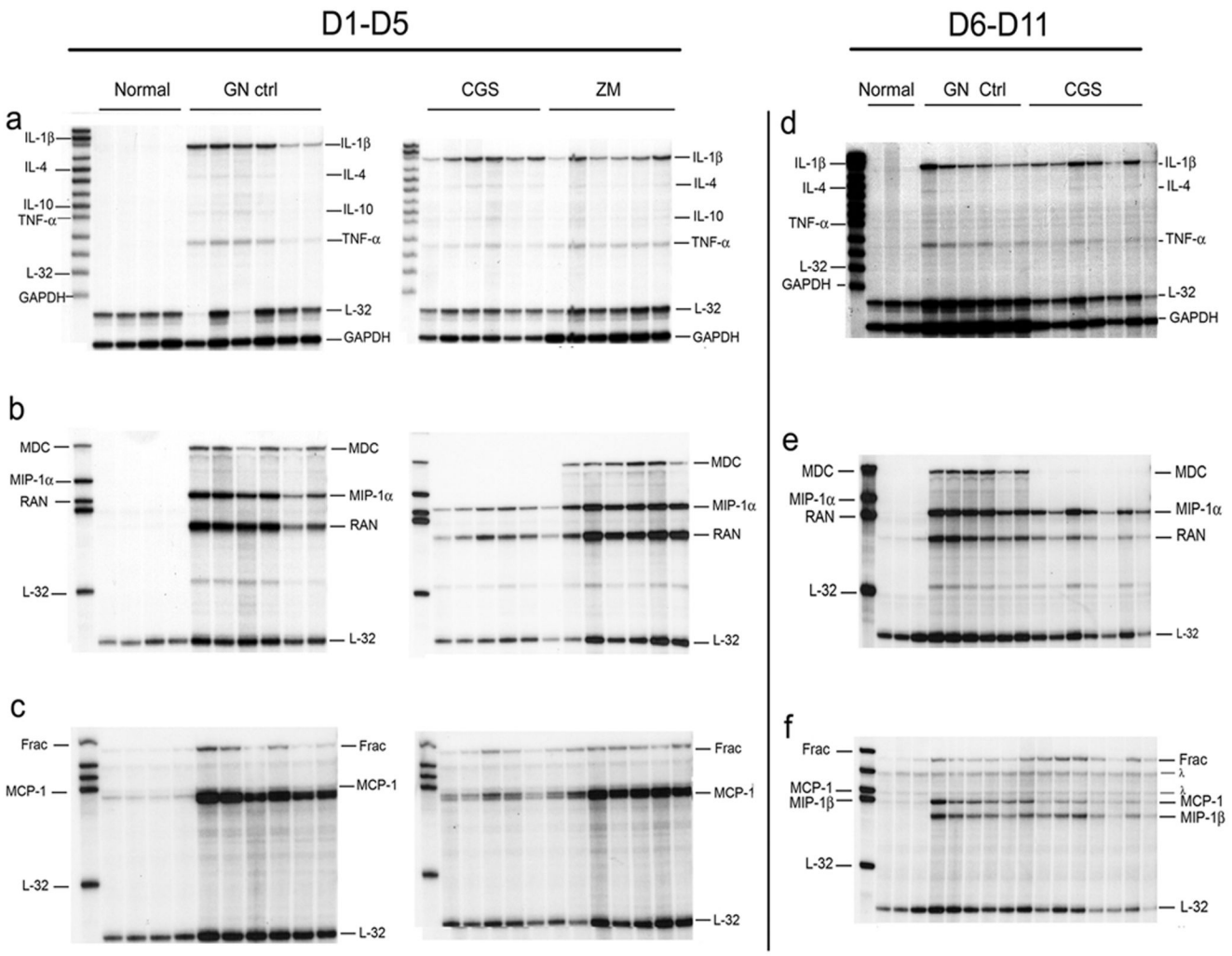

**4.**

$A_{2A}R$ activation decreases α-SMA expression and restores α-Actinin-4 expression in anti-GBM GN. Immunohistochemistry stained for α-SMA and α-Actinin-4 of kidney sections of normal, control, CGS216809 (CGS), and ZM241385 (ZM)-treated rats with anti-GBM glomerulonephritis. In the acute phase of the disease (day 1 to day 5), the control group of glomerulonephritis rats display increased expression of α-SMA and lost of α-Actinin-4. In CGS-treated rats, the expression of α-SMA was reduced and the expression of α-Actinin-4 restored. Increased expression of α-SMA and enhanced loss of α-Actinin-4 was observed in rats treated with ZM. In the progressive phase of the disease (day 6 to day 11) CGS markedly reduced α-SMA and restored α-Actinin-4.



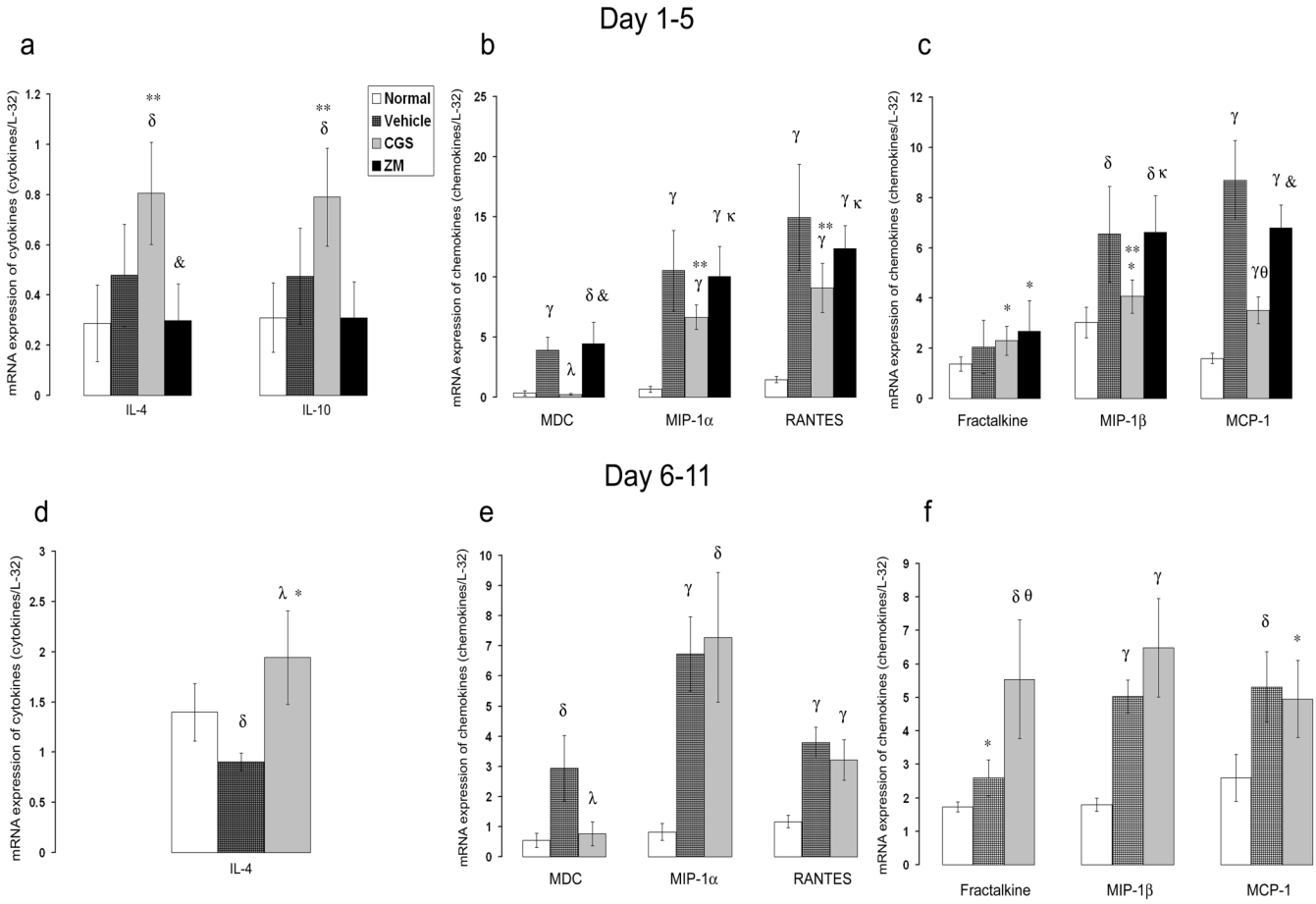

**5.**

Determination of proteinuria (milligrams of urine protein per 24 h) in Wistar-Kyoto rats with anti-GBM glomerulonephritis that were treated with vehicle, CGS21680 (CGS), and ZM241385 (ZM). In the acute phase of the disease, CGS completely blocked proteinuria in anti-GBM antibody-injected rats. Proteinuria was significantly higher in ZM-treated rats than the control group. In the progressive phase of glomerulonephritis, CGS markedly reduced proteinuria. Treatment Samples were sampled from six rats per group and expressed as mean ± SEM. *$p<0.0001$ vs. control, **$p<0.005$ vs. control, ***$p<0.05$ vs. control, $\delta p<0.005$ vs. ZM, &$p<0.0001$ vs. ZM



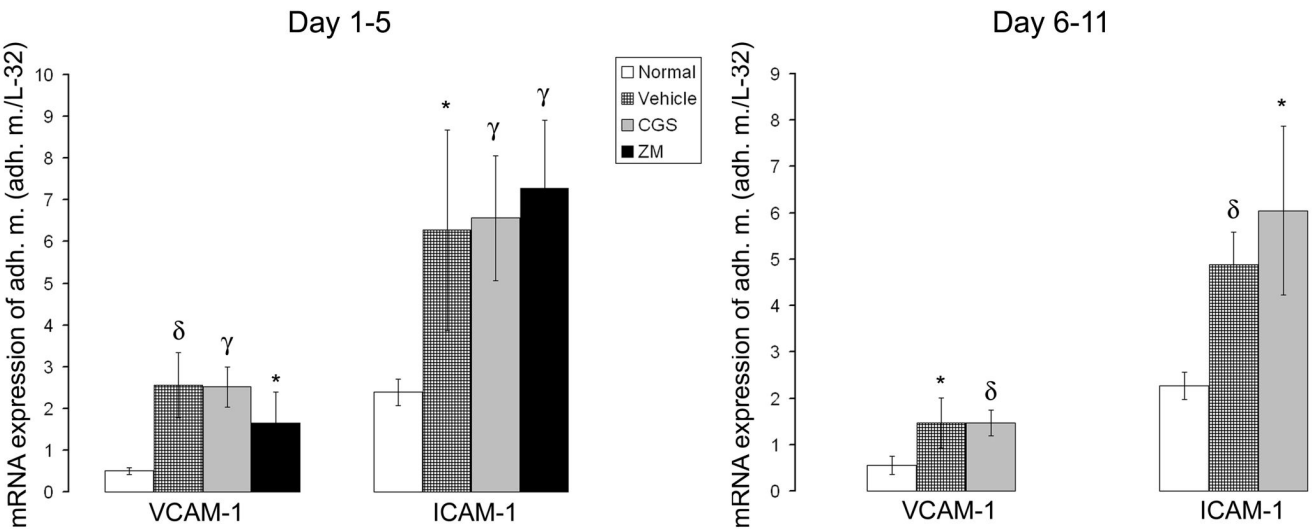

**6.**

Selective $A_{2A}R$ activation alters anti-GBM glomerulonephritis-induced cytokine expression. RNase protection assay of cytokines and chemokines expressed in the glomeruli of anti-GBM glomerulonephritis in WKY rats. CGS in the acute inflammatory phase of the disease (day 1 to day 5) increased the expression of IL-4 and IL-10 (a), abolished the increase of the expression of MDC/CCL22, and attenuated the expression of MIP-1α/CCL3, RANTES/CCL5, MIP-1β/CCL4, and MCP-1/CCL2 (b,c). ZM reduced IL-4 and IL-10 expression compared with CGS group (a). In the progressive phase of the disease (day 6 to day 11), CGS significantly enhanced the expression of IL-4 (d) and suppressed the increase of the expression of MDC/CCL22 (e). λ Refers to undigested probe. Each lane represents a single rat.




7.

Selective $A_{2A}R$ activation alters anti-GBM glomerulonephritis-induced cytokine expression. Densitometric analysis of blots from RNase protection assay of cytokines and chemokines expressed in the glomeruli of anti-GBM glomerulonephritis in WKY rats. The data are presented as a ratio of the cpm for the specific mRNA/L-32 mRNA to ensure a constant quantity of RNA in each sample. Results were sampled from six rats per group and expressed as mean ± SD. *$p<0.05$ vs. nl, δ$p<0.005$ vs. nl, γ$p<0.0005$ vs. nl, **$p<0.05$ vs. ctrl, θ$p<0.005$ vs. ctrl, λ$p<0.0005$ vs. ctrl, κ$p<0.05$ vs. CGS, &$p<0.005$ vs. CGS. nl refers to normal rat.




**8.**

Expression of adhesion molecules is not modified by pharmacological activation of $A_{2A}R$. Densitometric analysis of blots from RNase protection assay of VCAM-1 and ICAM-1 expressed in the glomeruli in anti-GBM glomerulonephritis. The data are presented as a ratio of the cpm for the specific mRNA/L-32 mRNA to ensure a constant quantity of RNA in each sample. Results were sampled from six rats per group and expressed as mean ± SD. *$p<0.05$ vs. nl, δ$p<0.005$ vs. nl, γ$p<0.0005$ vs. nl,