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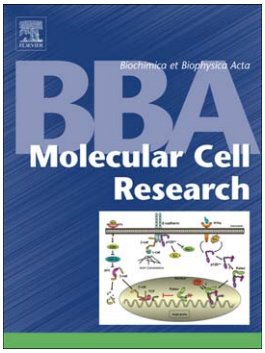

Adenosine A2A receptor signaling attenuates LPS-induced pro-inflammatory cytokine formation of mouse macrophages by inducing the expression of DUSP1

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Adenosine A2A receptor signaling attenuates LPS-induced pro-inflammatory cytokine formation of mouse macrophages by inducing the expression of DUSP1

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Abbreviations used in this paper: A2AR, adenosine A2A receptor; AP-1, activator protein 1, BLC, B-lymphocyte chemoattractant ; BMDM, bone marrow derived macrophage; CREB, cAMP response element-binding protein; DUSP1, dual specific phosphatase; Epac, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; HPRT, Hypoxanthine-guanine phosphoribosyltransferase ; hsp, heat shock protein; IFN, interferon; IKK, IκB kinase; IP-10, γ interferon-inducible cytokine; IκB, inhibitor of κ light chain gene enhancer in B cells; IL, interleukin; IRF, interferon regulatory factor; JNK, c-Jun N-terminal kinase; KC. keratinocyte chemoattractant; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MIP, macrophage inflammatory protein; MSK, mitogen and stress activated kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKA, cAMP-dependent protein kinase; PP, protein phosphatase; RSK, ribosomal S6 kinase; TIMP-1, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; TOR, target of rapamycin

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Highlights

- LPS stimulation of A2AR null macrophages results in a higher pro-inflammatory response
- Loss of A2ARs does not affect the LPS-induced NF-κB signaling pathway in macrophages
- Loss of A2ARs results in higher basal and LPS-induced MAPK phosphorylation
- A2AR signaling does not decrease MKK activation, but maintains high DUSP1 expression
- DUSP1 levels are regulated by A2ARs via the adenylate cyclase signaling pathway

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Abstract

Adenosine is known to reduce inflammation by suppressing the activity of most immune cells. Previous studies have shown that lipopolysaccharide (LPS) stimulated mouse macrophages produce adenosine, and the adenosine A2A receptor (A2AR) signaling activated in an autocrine manner attenuates LPS-induced pro-inflammatory cytokine formation. It has been suggested that A2AR signaling inhibits LPS-induced pro-inflammatory cytokine production through a unique cAMP-dependent, but PKA- and Epac-independent signaling pathway. However, the mechanism of inhibition was not identified so far. Here we report that LPS stimulation enhances A2AR expression in mouse bone marrow derived macrophages, and loss of A2ARs results in enhanced LPS-induced pro-inflammatory response. Loss of A2ARs in A2AR null macrophages did not alter the LPS-induced NF-κB activation, but an enhanced basal and LPS-induced phosphorylation of MAP kinases (especially that of JNKs) was detected in A2AR null cells. A2AR signaling did not alter the LPS-induced phosphorylation of their upstream kinases, but by regulating adenylate cyclase activity it enhanced the expression of dual specific phosphatase (DUSP)1, a negative regulator of MAP kinases. As a result, lower basal and LPS-induced DUSP1 mRNA and protein levels can be detected in A2AR null macrophages. Silencing of DUSP1 mRNA expression resulted in higher basal and LPS-induced JNK phosphorylation and LPS-induced pro-inflammatory cytokine formation in wild type macrophages, but had no effect on that in A2AR null cells. Our data indicate that A2AR signaling regulates both basal and LPS-induced DUSP1 levels in macrophages via activating the adenylate cyclase pathway.

Key words: lipopolysaccharide, macrophages, adenosine A2A receptor, cAMP, dual specific phosphatase-1

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1. Introduction

Adenosine is a purine nucleoside that, following its release from cells or after being formed extracellularly, diffuses to the cell membrane of surrounding cells, where it binds to its receptors [1,2]. There are four adenosine receptors, all of which are G protein-coupled and are abundantly expressed by macrophages [3]. The genes for these receptors have been analyzed in detail and are designated A_1, A_{2A}, A_{2B} and A_3. Adenosine A_1 receptors are stimulated by 10^{-10}-10^{-8} M concentrations of adenosine and mediate decreases in intracellular cyclic AMP (cAMP) levels, adenosine A_{2A} and A_{2B} receptors are stimulated by higher (5×10^{-7} M and 1×10^{-5} M, respectively) concentrations of adenosine and mediate increases in cAMP levels, while adenosine A_3 receptors are stimulated by 10^{-6} M concentrations of adenosine and mediate adenylate cyclase inhibition. However, the response of adenosine receptors is also determined by their cell surface expression, thus, when one compares ligand potencies to modulate cAMP levels at comparative receptor densities, it is observed that adenosine is nearly equipotent at A_1, A_{2A}, and A_3 receptors but is some 50 times less potent at A_{2B} receptors [4]. Although adenosine is constitutively present in the extracellular space at low concentrations (<1 μM), its concentration can dramatically increase under inflammatory conditions either by formation intracellularly and export via transporters, or formation in the extracellular space from adenine nucleotides released from cells [5] reaching concentrations high enough [6,7] to exert immunomodulatory and especially immunosuppressive effects [3]. Previous studies have shown that adenosine among many others inhibits lipopolysaccharide (LPS)-induced pro-inflammatory cytokine formation in monocytes and macrophages acting via adenosine A_{2A} receptors (A2ARs) [8-12].

LPS signaling regulates the expression of pro-inflammatory cytokines in macrophages via Myd88-dependent and independent pathways by influencing the activity of basically three transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB),

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activator protein 1 (AP-1) and interferon regulatory factors (IRFs) [13]. NF-κB and IRF5 are regulated via the IKK (IκB kinase), while AP-1 via the mitogen-activated protein kinase (MAPK) pathways. IKKs phosphorylate IκB (inhibitor of κ light chain gene enhancer in B cells) proteins. This phosphorylation leads to the degradation of IκB proteins and the subsequent translocation of the transcription factor NF-κB into the nucleus[14]. IKKβ also phosphorylates IRF5 directly leading to its activation [15]. AP-1, on the other hand, is regulated via direct phosphorylation by various mitogen-activated protein kinases (MAPK) including p38 and jun kinases (JNK) [16].

LPS stimulation in macrophages also results in ATP release via so far unidentified ATP channels leading to CD39-dependent ATP degradation and adenosine production [17] and in increases in the expression of the adenosine A2ARs via activation of the NFκB transcription factor. Since adenosine interferes with LPS signaling, up-regulation of the A2AR signaling is part of a delayed feedback mechanism initiated through NF-κB to terminate the activation of human and mouse macrophages [12,18,19]. The inhibition is mediated via cAMP and a non-characterized protein phosphatase, but is independent of protein kinase A or exchange protein activated by cAMP (Epac) [20]. However, the mechanism, through which adenosine interferes with the LPS signaling in macrophages has not been identified yet. In the present study we decided to further characterize the signaling pathway through which A2ARs inhibit LPS-induced pro-inflammatory cytokine formation by studying A2AR null macrophages.

2. Materials and Methods

2.1. Reagents

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All reagents were obtained from (Sigma-Aldrich, Budapest) except indicated otherwise.

2.2 Cell culture

Bone marrow-derived macrophages (BMDMs) were isolated from femurs of 3 to 6 months old wild type and A_{2A} receptor null mice generated on FVB background [21] after being killed by ether anesthesia. Macrophages were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin and 10% L929 conditioned media for 10 days. The non-adherent cells were washed away from the 3rd day daily. Study protocols were approved by the Animal Care Committee of the University of Debrecen [DEMÁB].

2.3. Flow cytometry

5×10^5 BMDMs were treated with 200 ng/ml LPS for the indicated time periods. After the incubation, macrophages were washed (1× PBS), collected, blocked with 50% FBS for 30 min, and labeled with anti-mouse A2AR antibody (BD Pharmingen) or goat IgG isotype control. For detection, cells were stained with FITC-conjugated anti-goat IgG. Stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

2.4. Determination of cytokine production

$5x10^5$ wild type and A2AR null BMDMs were treated with 200 ng/ml crude LPS for 1 h. In some experiments, cells were pretreated with the A2AR-specific agonist CGS21680 (1 μM,

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Tocris), the A2AR-specific antagonist SCH442416 (10 nM, Tocris), the JNK inhibitor TCS JNK 60 (100 nM), Rp-cAMPs (100 μM) or forskolin (10 μM) for 1 h. After 1 h LPS was removed and fresh medium was added to the cells. Where it is indicated, media was supplemented with the compounds used in the pretreatments. 5 h later supernatants were collected, centrifuged and analyzed by Mouse Cytokine Array (Proteome Profile Array from R&D Systems) or by interleukin (IL)-6, macrophage inflammatory protein (MIP)2, tumor necrosis factor (TNF)-α ELISA kits (R&D Systems) according to the manufacturer's instructions. In case of cytokine array, the pixel density in each spot of the array was determined by Image J software.

2.5. Quantitative RT-PCR

After various treatments 2×10^6 BMDMs were washed with ice-cold PBS. RNA was extracted with Tri-reagent. cDNA was synthesized with High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instruction. Hypoxanthine-guanine phosphoribosyltransferase (HPRT), macrophage inflammatory protein (MIP-2), A2AR, IL-6, tumor necrosis factor (TNF)-α and dual specific phosphatase (DUSP)1 levels were determined with Taq-Man PCR using FAM-MGB labeled probes (Applied Biosystems) by Real-time PCR on Roche LightCycler 480 platform. Samples were run in triplicate. Gene expression was normalized to HPRT expression

2.6. Western blot

2×10^6 wild-type and A2AR null BMDMs were treated with 200 ng/ml LPS for the indicated time periods. Where it is indicated, cells were pretreated with 1 μM CGS21680 or 10 nM

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SCH442416 for 1 h. Cells were harvested at the indicated time points and boiled 2x sample buffer and loaded onto SDS PAGE gels. PVDF membranes were probed with anti-IκBα (Santa Cruz Biotechnology), anti-MKP1 (Santa Cruz Biotechnology), anti-phospho(Thr183/Tyr185)-SAPK/JNK (Cells Signaling Technology), anti-lamin B (Santa Cruz Biotechnology), anti-mitogen-activated kinase kinase (MKK) 3/4 pMKK3/4/6 (Cell Signaling and Technology) and anti-histone H2B (Santa Cruz Biotechnology). The pixel density of bands was determined by Image J software.

2.7. Determination of NF-κB p50/p65 nuclear translocation

10^7 wild-type and A2AR null BMDMs were treated with 200 ng/ml LPS for 60 min. Where it is indicated, cells were pretreated with A2AR-sepcific agonist 1 μM CGS21680 for 1 h. Cell were rinsed with ice cold PBS and nuclei were isolated with Nuclei EZ kit (Sigma) according to manufacturer's instruction. Nuclear p65 and p50 subunits were detected with TransAM p65 and p50 kits (ActiveMotif) according to manufacturer's instruction.

2.8. Determination of NFκB-dependent transcription

NFκB-dependent transcription was evaluated as luciferase activity derived from the expression of an NFκB-dependent luciferase transcriptional reporter in a stably transfected macrophage cell line, H2 [22]. H2 cells were plated (4×10^5 cells/well) in a 24-well plate. The next day, 125 ng/ml LPS and the indicated doses of CGS21680 were added simultaneously. After incubation for 6 h, cell extracts were prepared, and luciferase activity was measured by the Luciferase Assay System (Promega) in an FB12 Luminometer (Zylux). Each condition

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was repeated in duplicate wells, and the luciferase activities in cells from each well were determined independently.

2.9. Determination of MAPK phosphorylation

$1x10^6$ cells/well BMDMs treated with 200 ng/ml LPS for 1 h. Where it is indicated, cells were pretreated with 10 nM SCH442416 (10 nM, Tocris) for 1 h. After 30 min LPS treatment, cells were rinsed with PBS. Total cell lysates were analyzed by Human Phospho-MAPK Array Kit (R&D Systems) according to manufacturer's instruction. The pixel density in each spot of the array was determined by ImageJ software.

2.10. Determination of panJNK phosphorylation by ELISA method

$2x10^6$ wild-type and A2AR null BMDMs were treated with 200 ng/ml LPS for 30 min. In some case, cells were pretreated with pretreated with 1 μM CGS21680 or 10 nM SCH442416 for 1 h. After the LPS treatment, cells were rinsed with PBS and total cell lysates were analyzed by Phospho-JNK Pan Specific DuoSet IC MAPK ELISA (R&D Systems).

2.11. DUSP1 siRNA transfection

Five-day-matured BMDMs were transfected with ON TARGETPLUS SMARTpool siRNA specific for mouse DUSP1 and ON-TARGETPLUS Non-targeting Control Pool (Dharmacon) using the DharmaFECT 1 Transfection Reagent (Dharmacon) according to the DharmaFECT's Transfection Protocol. siGLO Green (6-FAM) Transfection Indicator was used to monitor the transfection efficiency. Transfected cells were used 5 days after the

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transfection. The efficiency of RNAi was determined by Western blot and quantitative PCR against DUSP1.

2.12. Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± S.D. P values were calculated by using two-tailed Student's t-test for two samples of unequal variance. The analysis of cytokine and MAPK array experiments was carried out by two-way ANOVA test. Statistical calculations were run on GraphPad Prism 6 software. Statistical significance is indicated by asterisk *(*p<0.05).*

3. Results

3.1. Adenosine A2A receptors mediate a feedback regulatory mechanism to decrease the LPS-induced pro-inflammatory cytokine formation in bone marrow derived macrophages

Previous studies have indicated that exposure of macrophages to LPS results in enhanced expression of A2ARs on macrophages [18,19]. As it is shown in Figure 1, LPS indeed induced the expression of A2ARs on both on the cell surface (Fig.1A) and on mRNA levels (Fig. 1B) in BMDMs. Since the increase in the cell surface expression of A2ARs could be detected already at 30 min, it is very likely that not only the reported transcriptional mechanisms [18,19] contribute to its enhanced expression.

To investigate the involvement of A2ARs in the regulation of LPS-induced pro-inflammatory cytokine formation, both wild type and A2AR null macrophages were exposed

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to LPS for 1 h, and the pro-inflammatory cytokine production was determined after an additional 5 h time period by using a cytokine array (Fig.1C). As shown in Figure 1D, loss of A2ARs affected already the basal pro-inflammatory cytokine production of BMDMs, A2AR null macrophages producing more B-lymphocyte chemoattractant (BLC), interferon (IFN)γ, γ interferon-inducible cytokine (IP-10), keratinocyte chemoattractant (KC) and tissue inhibitor of metalloproteinases (TIMP)-1. Following LPS exposure macrophages started to release numerous pro-inflammatory cytokines and the production of all of these was found to be enhanced by A2AR null cells. The results of cytokine array were further confirmed by ELISA for the production of TNF-α, IL-6 and MIP-2 (Fig. 1E). Not only the protein, but also the mRNA expression of these cytokines was higher in LPS-exposed A2AR null cells as compared to the wild- type cells (Fig. 1F). These data confirm previous findings that LPS-exposed macrophages produce endogenous adenosine to control LPS-induced pro-inflammatory cytokine formation via the adenosine A2ARs [8-12].

3.2. Loss of adenosine A2A receptors does not affect the LPS-induced NF-κB signaling pathway in macrophages

Since the signalling pathways induced by LPS transduce their effect on pro-inflammatory cytokine production partly via activating NF-κB, and these pathways regulate the degradation of the inhibitory subunit IκBα [23], a negative regulator of NF-κB, we decided to determine the IκBα levels in wild-type and A2AR null macrophages following LPS stimulation. As shown in Figure 2A, neither the amount, nor kinetics of the IκBα degradation induced by LPS stimulation were found to be different in the two cell types. Similar was the finding if wild type macrophages were pre-treated for 1 h with CGS21680, an A2AR agonist (Fig. 2B).

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NF-κB is a dimeric transcription factor that belongs to the Rel-homology domain-containing protein family, which includes p65/RelA, p50/NF-κB1, p52/NF-κB2, RelB and c-Rel. The prototypical NF-κB is thought to be a heterodimer composed of the p65 and p50 subunits in most types of cells [23]. Thus we decided to test whether the loss of A2AR affects the nuclear translocation of the p65/p55 subunit of NF-κB by using the TransAM NF-κB transcription factor kits (ActiveMotif). As shown in Figure 2C, there was no difference in either the basal or in the LPS-induced nuclear translocation of these transcription factors in wild type and in A2AR null macrophages. In line with these observations, administration of the A2AR agonist CGS21680 also did not alter it. Administration of CGS21680 did not alter the LPS-induced NF-κB-driven transcriptional activation of a luciferase construct in a stably transfected RAW264.7 mouse macrophage cell line [22] either (Fig. 2D). All together these data indicate that NF-κB is properly activated in the presence of adenosine, and not the NF-κB pathway is the main target of A2AR signalling through which it attenuates the LPS-induced pro-inflammatory cytokine formation. Thus we decided to study the MAPK signalling pathways.

3.3. Adenosine suppresses both basal and LPS-induced JNK activities acting via A2ARs

To test the basal and LPS-induced MAPK activation in the absence of A2AR signalling in macrophages, we applied a human phospho-MAPK array kit, which enables the parallel determination of relative levels of phosphorylation of MAPKs and several related proteins (Fig. 3A). As shown in Figure 3B upper panels, loss of A2ARs resulted in enhanced basal phosphorylation of p38α, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK)2. This phenomenon could be partially mimicked by a 1 h administration of the A2AR antagonist SCH442416 indicating that alteration in the basal phosphorylation pattern is

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not a result of a developmental defect of macrophages in the absence of A2ARs. This observation also indicated that A2ARs must be continually activated in non-stimulated cells.

Stimulation of wild-type macrophages by LPS (Fig. 3B lower panel left) resulted in activation of the MAPK pathways [16] leading to enhanced phosphorylation of p38, ERK2 and JNK and that of the downstream proteins of the p38 MAP kinase pathway such as ribosomal S6 kinase (RSK) 1, 2, mitogen and stress activated kinase 2 (MSK2) [16]. Previous studies have shown that p38α can selectively activate MAPK-activated protein kinase 2, which further phosphorylate cAMP response element-binding protein (CREB) and heat shock protein (hsp) 27 [24], the activation of which was also detected. In addition, we could also demonstrate enhanced phosphorylation of proteins of the phosphatidylinositol-3 kinase pathway such as target of rapamycin (TOR) and glycogen synthetase kinase 3. This latter pathway has been suggested to limit the LPS-induced signaling pathways by inhibiting the LPS-induced nuclear translocation of NF-κB [25].

While loss or inhibition of A2ARs resulted in enhanced basal phosphorylation of the p38α and ERK2, the phosphorylation of these kinases did not increase further following LPS stimulation in the absence of A2AR signaling (Fig.3B lower middle and right panel). However, despite of the lack of further phosphorylation, following LPS exposure the phosphorylation of the p38α MAP kinase was still higher in A2AR null macrophages than in wild type cells. In contrast, after LPS stimulation the phosphorylation of JNKs increased significantly further in A2AR null macrophages as well (Fig. 3B lower middle panel). Since macrophages express JNK1 and 2 [26], but the human phospho-MAPK array kit did not detect mouse JNK1, we tested its phosphorylation by Western blot analysis by using a mouse specific anti-JNK1 antibody. As shown in Figure 3C, phosphorylation of JNK1 was also enhanced following LPS stimulation in A2AR null macrophages as compared to their wild counterparts. The findings were further confirmed by using a phospho-panJNK-specific

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ELISA kit (Fig. 3D). In accordance, LPS-induced phosphorylation of JNKs in wild type cells was further enhanced when these cells were exposed to SCH442416, while was inhibited when exposed to CGS21680 (Fig. 3D). These data indicated that A2AR signaling might negatively influence JNK activity. Thus, we decided to test the potential involvement of JNKs in the A2AR-mediated attenuation of LPS signaling in macrophages.

3.4. JNK is a downstream target in the control of LPS-induced pro-inflammatory cytokine formation by adenosine

If A2AR signaling affects LPS-induced pro-inflammatory cytokine formation via decreasing the LPS-induced phosphorylation of JNK, then inhibition of JNK should affect the ability of adenosine to control LPS-induced pro-inflammatory cytokine formation. As shown in Figure 4, exposure of wild type macrophages to CGS21680 attenuated, while that of SCH442416, enhanced the LPS-induced production of IL-6 (Fig. 4A), MIP-2 (Fig. 4B) and TNF-α (Fig. 4C). The same compounds did not affect the LPS-induced pro-inflammatory cytokine production of A2AR null macrophages confirming the A2AR selectivity of these compounds. In the presence of the A2AR antagonist a similarly high LPS-induced pro-inflammatory cytokine production was found by wild type macrophages, as by A2AR null cells.

Inhibition of JNK activity by TCS-JNK attenuated LPS-induced pro-inflammatory cytokine production to a similar degree in wild type macrophages, as in their A2AR null counterparts. In addition, those wild-type macrophages, in which JNK activity was inhibited, failed to respond to A2AR signaling in the regulation of LPS-induced pro-inflammatory cytokine production. All together these data indicate that JNK is a downstream target of adenosine signaling.

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3.5. A2AR signaling enhances both basal and LPS-induced DUSP1 expression via the adenylate cyclase pathway

Enhanced phosphorylation of MAPKs in A2AR null macrophages might be the result of either an enhanced activation of their upstream kinases, or a decreased removal of their phosphate groups by their specific phosphatases. Since we detected activation of both p38α and JNKs, downstream elements of LPS-induced MKK4 or MKK3/6 activation [24,27], we checked whether MKK4 or MKK3/6 activation is increased by LPS in the absence of A2AR signaling. However, as shown in Fig. 5A, in the case of MKK4 no alteration was found in the kinetics phosphorylation. In addition, following LPS exposure both the levels of MKK3 and the phosphorylation of MKK3/6 decreased in the presence of the A2AR antagonist. These observations indicate that not an enhanced activation of the upstream kinases in the LPS signaling pathway is responsible for the enhanced MAP kinase phosphorylation observed in the absence of A2AR signaling.

During the last decade, a family of dual specificity phosphatases was identified that acts as MAPK phosphatases by dephosphorylating them at threonine and tyrosine residues. The prototypic member of this family is DUSP1, which is expressed by macrophages, and the expression of which is enhanced during LPS stimulation [28, 29]. The enzyme is an important negative-feedback regulator of macrophage function and the inflammatory response to TLR signaling, and plays key regulatory roles in innate immune responses via inactivation of p38 and JNK [30,31]. Since already the basal phosphorylation level of both JNK and p38 was enhanced in A2AR null macrophages (Fig. 3B), we decided to determine whether basal DUSP1 levels are affected by the loss of A2AR. As shown in Figure 5, A2AR null macrophages express significantly lower DUSP1 at both mRNA (Fig. 5B) and protein (Fig. 5C) levels than their wild type counterparts. Exposure of wild type macrophages to SCH442416 also resulted in a decrease in DUSP1 expression (Fig. 5D and E), while that to

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CGS21680 increased it (Fig. 5F and G). These compounds did not affect DUSP1 levels in A2AR null cells indicating that they acted indeed via A2ARs. All together these data indicate that A2AR signaling continuously enhances the basal DUSP1 expression in BMDMs.

Previous studies have shown that LPS stimulation itself enhances the expression of DUSP1 in macrophages, which contributes to termination of the LPS response [28]. Thus we decided to investigate whether A2AR signaling affects LPS-regulated DUSP1 expression. For this purpose macrophages were pretreated for 1 h with either the A2AR agonist or the A2AR antagonist, and DUSP1 mRNA expressions were determined. The time point was set for 2h following LPS treatment, as preliminary studies revealed that the LPS-induced DUSP1 mRNA expression is the highest at this time point (data not shown). As shown in Figure 5H, not only the basal, but also the LPS-induced DUSP1 mRNA expressions were higher in wild type macrophages as compared to that of the A2AR null cells. In line with this finding, in wild type cells addition of the A2AR agonist enhanced, while that of the A2AR antagonist decreased the LPS induced DUSP1 mRNA expression.

Previous studies have shown that A2ARs mediate their effect on the LPS signaling in macrophages by elevating intracellular cAMP levels [20]. To investigate whether alterations in intracellular cAMP levels affect DUSP1 mRNA levels, macrophages were pretreated for 1 h with either forskolin, an adenylate cyclase activator [32], or Rp-cAMP, a competitive inhibitor of endogenous cAMP. As shown in Figure 5H, forskolin could enhance both the basal and the LPS-induced DUSP1 expression in both wild type and A2AR null macrophages, while Rp-cAMP decreased both the basal and the LPS-induced DUSP1 mRNA levels, but only in wild type cells. In addition, Rp-cAMP prevented the enhancing effect of the A2AR agonist on DUSP1 levels, while forskolin the decreasing effect of the A2AR antagonist on DUSP1 levels in wild type cells. All together these data indicate that A2ARs upregulate the

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mRNA levels of DUSP1 in both resting and in LPS-induced macrophages acting via the adenylate cyclase pathway.

3.6. A2ARs fail to control the LPS-induced JNK phosphorylation in DUSP1-silenced macrophages

To prove the involvement of DUSP1 in A2AR signaling, DUSP1 levels were silenced in both wild type and in A2AR null macrophages. As shown in Figure 6A, siRNAs significantly reduced the basal DUSP1 expression at both protein and mRNA levels in wild type and A2AR null macrophages. Reduction in DUSP1 levels by siRNA resulted in an enhancement in both the basal and in the LPS-induced phosphorylation levels of JNK in wild type macrophages, and in a loss of the A2AR control over it (Fig. 6B). In contrast, in A2AR null cells reduction in DUSP1 levels had only slight effect on the enhanced basal and LPS-induced JNK phosphorylation (Fig. 6B). These data indicate that A2ARs regulate JNK phosphorylation via modifying the expression of DUSP1.

3.7. A2ARs fail to control the LPS-induced IL-6 and TNF-α production in DUSP1-silenced macrophages

To investigate further the involvement of DUSP1 in the A2AR-mediated control of LPS-induced pro-inflammatory cytokine formation, the effect of DUSP1 silencing was studied on the LPS-induced IL-6 (Fig. 6C) and TNF-α (Fig. 6D) production of wild type and A2AR null macrophages. In wild type macrophages, silencing of DUSP1 enhanced the LPS-induced production of each of these three pro-inflammatory cytokines. DUSP1 silenced cells became resistant to A2AR signaling, since neither CGS21680, nor SCH442416 affected their LPS-induced pro-inflammatory cytokine formation. In contrast, silencing of DUSP1 only slightly

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affected the LPS-induced enhanced production of these pro-inflammatory cytokines in A2AR null cells. These data indicate that A2ARs regulate LPS-induced IL-6 and TNF-α production via controlling DUSP1 levels in macrophages.

4. Discussion

Innate responses orchestrate the immediate and early phases of host defense to microbes as well as to injury, initiating the inflammatory reaction and recruiting cells of the acquired immune system to the site of inflammation. Although inflammatory responses against invading microbial pathogens are critical mechanisms for survival, dysregulated inflammatory responses are detrimental to the host. Thus immune systems have evolved multiple strategies to regulate and maintain an adequate level of inflammation including induction of negative feedback regulators for inflammation, such as production of adenosine [17]. Despite of the fact that it has been known for a long time that adenosine suppresses LPS-induced pro-inflammatory cytokine formation, no mechanism has been found so far to explain the phenomenon. It is generally believed that A2AR activation inhibits the LPS-induced expression of pro-inflammatory mediators by elevating the intracellular concentration of cAMP, which subsequently inhibits NF-κB activity and thereby gene transcription through a signaling mechanism involving cAMP dependent protein kinase A [33,34]. However, later studies indicated that though inhibition of LPS-induced pro-inflammatory cytokine formation by adenosine is mediated by cAMP, it does not involve protein kinase A or Epac [20], and NF-κB activity is not affected [20,35,36]. In line with these latter observations, we also found that LPS-regulated NF-κB activation is not affected by A2AR signaling in macrophages. Instead, we found that A2AR signaling is continually activated in macrophages and suppresses MAPK activities. Consequently, in A2AR null macrophages the basal

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phosphorylation levels of p38α, ERK2 and JNK MAP kinases were found to be higher than in their wild type counterparts. Following LPS stimulation the A2AR levels are increased in macrophages, and adenosine signaling suppressed also the LPS-induced MAP kinase activation. As a result, JNK phosphorylation and signaling were attenuated and the LPS-induced expression of JNK-dependent pro-inflammatory cytokines, such as MIP-2, IL-6 and TNF-α, was found to be attenuated. While JNK enhances the amounts of MIP-2 [37] and IL-6 [38] primarily via regulating transcription, expression of TNF-α was shown to be regulated by JNK at translational level [39]. Though we did not investigate the consequences of the adenosine-induced p38 MAP kinase or ERK suppression in LPS-stimulated macrophages, these pathways are also known to contribute to the formation of various pro-inflammatory cytokines. Thus, for example just in the context of TNF-α, it was shown that A2AR signaling suppresses p38 activity leading to TNF-α mRNA instability in U937 cells [40], while ERK is required for LPS-induced TNF-α mRNA production in macrophages [41].

MAP kinase activity can be regulated by upstream kinases that phosphorylate MAP kinases at both serine and threonine residues, and by dual specific phosphatases, which dephosphorylate and inactivate these enzymes. While we have not found an increase in the activation of MKK4 and MKK3/6, upstream activators of p38 MAP kinases and JNKs, we have demonstrated that the expression of at least one of the MAPK phosphatases, DUSP1, is positively regulated by the A2AR-induced adenylate cyclase signaling pathway. The mechanism of DUSP1 upregulation was not investigated in our studies. DUSP1 expression is known to be induced by phosphorylated CREB, a target of protein kinase A [42]. In our present studies the involvement of protein kinase A was not addressed, but if A2AR signaling is indeed independent of protein kinase A [20], CREB is not likely to be involved. Altogether our data indicate that macrophages continually produce adenosine to activate A2ARs, which

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trigger a cAMP-mediated pathway in macrophages to maintain the expression of DUSP1 and consequently to suppress both the basal and the LPS-induced MAP kinase activities.

Conflict of interest

We have no conflict of interest in any part of this article.

Acknowledgements

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Legends to the Figures

Fig. 1. Loss of A2ARs results in higher pro-inflammatory cytokine formation in LPS-stimulated bone marrow derived macrophages. A2AR expression is induced in response to LPS-stimulation both (A) on the cell surface and (B) at mRNA expression level in BMDMs. Wild-type BMDMs were treated with 200 ng/ml LPS for the indicated time periods, and cell surface expression of A2ARs was determined by flow cytometry, while gene expression levels were measured by qRT-PCR using HGPRT as a normalizing gene. The results are expressed as mean ± SD of five independent experiments (*significantly different from non-treated control, $p<0.05$). (C) The map of the 40 cytokines detected on the membranes. (D) Cytokine panel of control and LPS-treated wild-type and A2AR null BMDMs. Cells were incubated with 200 ng/ml LPS for 1 h. After the removal of LPS fresh media were added to the cells. Supernatants were collected 5 h later, and cytokine levels were determined by cytokine array. Arrows highlight those cytokines, the expression of which was significantly different in A2AR null BMDMs. The pixel density results are mean±S.D. from three independent experiments (*significantly different from wild-type control, $p<0.05$). Effect of LPS treatment on the TNF-α, IL-6 and MIP-2 production of wild type and A2AR null BMDMS at (E) protein and at (F) mRNA levels. Wild-type and A2AR null BMDMs were treated for with 200 ng/ml LPS for 1 h. After the removal of LPS fresh media were added to the cells. Supernatants were collected 5 h later and the levels of indicated cytokines were determined by ELISA method. Alternatively, total RNA was isolated after 3 h of incubation and cytokine gene expression levels were measured by qRT-PCR using HGPRT as normalizing gene. Relative gene expression values are shown as fold induction normalized to the wild-type control samples. Results are expressed as mean ± SD of six independent experiments (*significantly different from wild type control, $p<0.05$).

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Fig. 2. The enhancement in LPS-induced pro-inflammatory response of A2AR null macrophages is not related to an alteration in the activation of the classical, NF-κB-dependent TLR4 signaling pathway. Western blot analysis of IκBα degradation in wild-type and A2AR null macrophages after exposure to 200 ng/ml LPS alone (A) or in combination with A2AR-specific agonist CGS21680 (1 μM, 1h pre-treatment) (B) for the indicated time periods. Lamin-B was used as a loading control. (C) Nuclear levels of the p65 and p50 subunits of NF-κB transcription factor in control and LPS-stimulated macrophages. Wild-type and A2AR null BMDMs were treated with 200 ng/ml LPS for 1h. Nuclear levels of the p65 and p50 subunits of NF-κB were detected with TransAM NF-κB transcription factor kits. The results are expressed as mean ± SD of three independent experiments. (D) Activation of the A2AR signaling pathway does not affect the NFκB-driven transcriptional activity determined by measuring the luciferase activity of an NFκB-dependent luciferase transcriptional reporter in a stably transfected RAW264.7 macrophage cell line. 125 ng/ml LPS and the indicated doses of CGS21680 were added to the cells simultaneously. Cell extracts were prepared, and luciferase activities were measured after a 6h LPS exposure. Data were normalized to the non-treated control cells. Results are expressed as mean ± SD of three independent experiments.

Fig. 3. A2AR deficiency is accompanied by enhanced MAPK phosphorylation. (A) The map of the 26 MAPK cascade-associated proteins the phosphorylation of which can be detected on the Phospho-MAPK array membranes. (B) Phospho-MAPK panel of wild-type and A2AR null BMDMs with or without LPS treatment alone or in the presence of an A2AR antagonist. Wild-type and A2AR null cells were treated with 200 ng/ml LPS alone or in combination of t SCH442416 (10 nM, 1h pre-treatment) for 30 min. Macrophages were then lysed, and the amount of phosphorylated proteins was detected by a Phospho MAP kinase array. Arrows highlight those proteins, the phosphorylation of which was altered in A2AR null BMDMs as

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compared to their wild-type control. One representative experiment of three is shown. (C) Western blot analysis of JNK1 and phospho-JNK1 levels in wild-type and A2AR null macrophages exposed to 200 ng/ml LPS for the indicated time periods. Lamin B was used as a loading control. Integrated density values of the blot are shown on right. (D) ELISA-based determination of phospho-JNK levels. BMDMs were treated with 200 ng/ml LPS alone or in combination with the A2AR-specific agonist CGS21680 (1 μM, 1h pre-treatment) or the A2AR-specific antagonist SCH442416 (10 nM, 1h pre-treatment) for 30 min. The levels of phosphorylated JNKs were determined by a phospho-panJNK-specific ELISA kit. The results are expressed as mean ± SD of three independent experiments. (*significantly different from the LPS-treated wild type cells, $p<0.05$).

Fig. 4. The A2AR-mediated suppression of LPS-induced pro-inflammatory cytokine production requires JNKs. BMDMs were treated with 200 ng/ml LPS alone or in combination with CGS21680 (1 μM, 1h pre-treatment), SCH442416 (10 nM, 1h pre-treatment) or the JNK inhibitor TCS-JNK (100 nM, 1h pre-treatment) for 1h. After the removal of LPS, fresh media supplemented with CGS21680, SCH442416 or TCS-JNK were added to the cells. Supernatants were collected 5h later, and IL-6 (A), MIP-2 (B) and TNF-α (C) levels were determined by ELISA. The results are expressed as mean ± SD of three independent experiments (*significantly different, $p<0.05$).

Fig. 5. The mRNA and protein expression levels of DUSP1 are under the control of a continuously activated A2AR signaling pathway in BMDMs. (A) Western blot analysis of MKK4, MKK3 and phospho-MKK4, phospho-MKK3/6 levels in wild-type macrophages exposed to 200 ng/ml LPS for the indicated time periods. Where it is indicated, SCH442416

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(10 nM, 1h pre-treatment) was also added to the cells. Lamin B was used as a loading control. *nt: non-treated control sample.* Basal DUSP1 mRNA (B) and protein (C) levels in wild type and A2AR null BMDMs. Effect of an A2AR antagonist on the DUSP1 mRNA (D) and protein (E) levels in wild type and A2AR null BMDMs. Effect of an A2AR agonist on the DUSP1 mRNA (F) and protein (G) levels in wild type and A2AR null BMDMs. Total protein or total RNA of untreated BMDMs, or BMDMs exposed to either CGS21680 (1 μM, 3h treatment) or SCH442416 (10 nM, 3h treatment) were isolated. DUSP1 protein levels were analyzed by Western blot using histone H2B as a loading control. Alternatively, DUSP1 gene expression levels were determined by qRT-PCR using HGPRT as normalizing gene. Relative gene expression values are expressed as mean ± SD of four independent experiments (*significantly different $p<0.05$). (H) DUSP1 gene expression level of LPS-exposed wild-type and A2AR null BMDMs. Prior to LPS exposure (200 ng/ml, 1h treatment) cells were pre-treated with CGS21680 (1 μM), SCH442416 (10 nM), Rp-cAMPS, a competitive inhibitor of cAMP (100 μM) or forskolin, an adenylyl cyclase activator (10 μM). After the LPS treatment fresh media - supplemented with CGS21680, SCH442416, Rp-cAMPS, or forskolin - were added to the cells. Total RNA was isolated 2 h later, and DUSP1 gene expression levels were determined by qRT-PCR using HPGRT as normalizing gene. Results are expressed as fold induction as compared to the wild type non treated cells. Data show mean ± SD of three independent experiments (*significantly different, $p<0.05$).

Fig. 6. A2AR signaling is not able to regulate LPS-induced JNK activation and pro-inflammatory cytokine production in DUSP1-silenced BMDMs. (A) Transient DUSP1 silencing by siRNA. Five-day-matured BMDMs were transfected with siRNA specific for mouse DUSP1 and non-targeting siRNA. The efficiency of RNAi was determined by Western blot analysis and by qRT-PCR using HGPRT as normalizing gene. (B) Phosphorylated JNK (C) IL-6 and (D) TNF- α levels of LPS-treated wild-type and A2AR null DUSP1-silenced

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BMDMs in the presence of an A2AR agonist or antagonist. Control, non-targeting siRNA and DUSP1 siRNA transfected cells were treated with 200 ng/ml LPS alone or in combination with either CGS21680 (1 μM, 1h pre-treatment) or SCH442416 (10 nM, 1h pre-treatment) for 30 min for determining pJNK levels by a phospho-panJNK-specific ELISA kit, or for 1 h for the pro-inflammatory cytokine determination. After the removal of LPS fresh media supplemented with CGS21680 or SCH442416 were added to the cells. Supernatants were collected 5 h later, and IL-6 and TNF-α levels were determined by ELISA. Results are expressed as mean ± SD of three independent experiments (*significantly different from non-targeting siRNA, $p<0.05$).

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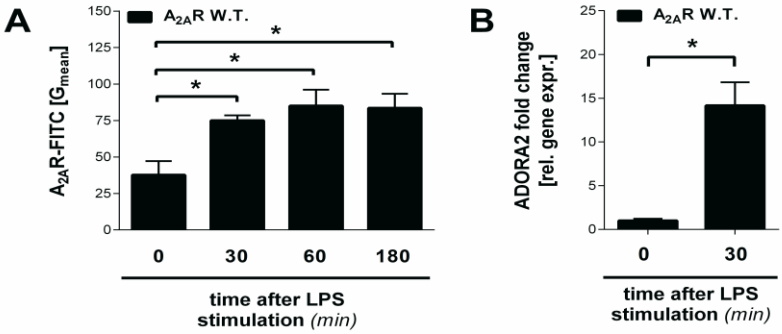

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B	BLC	C5a	G-CSF	GM-CSF	I-309	eotaxin	sICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12 p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1α	MIP-1β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF-α	TREM-1	-	-	-	-	-	-	-	-
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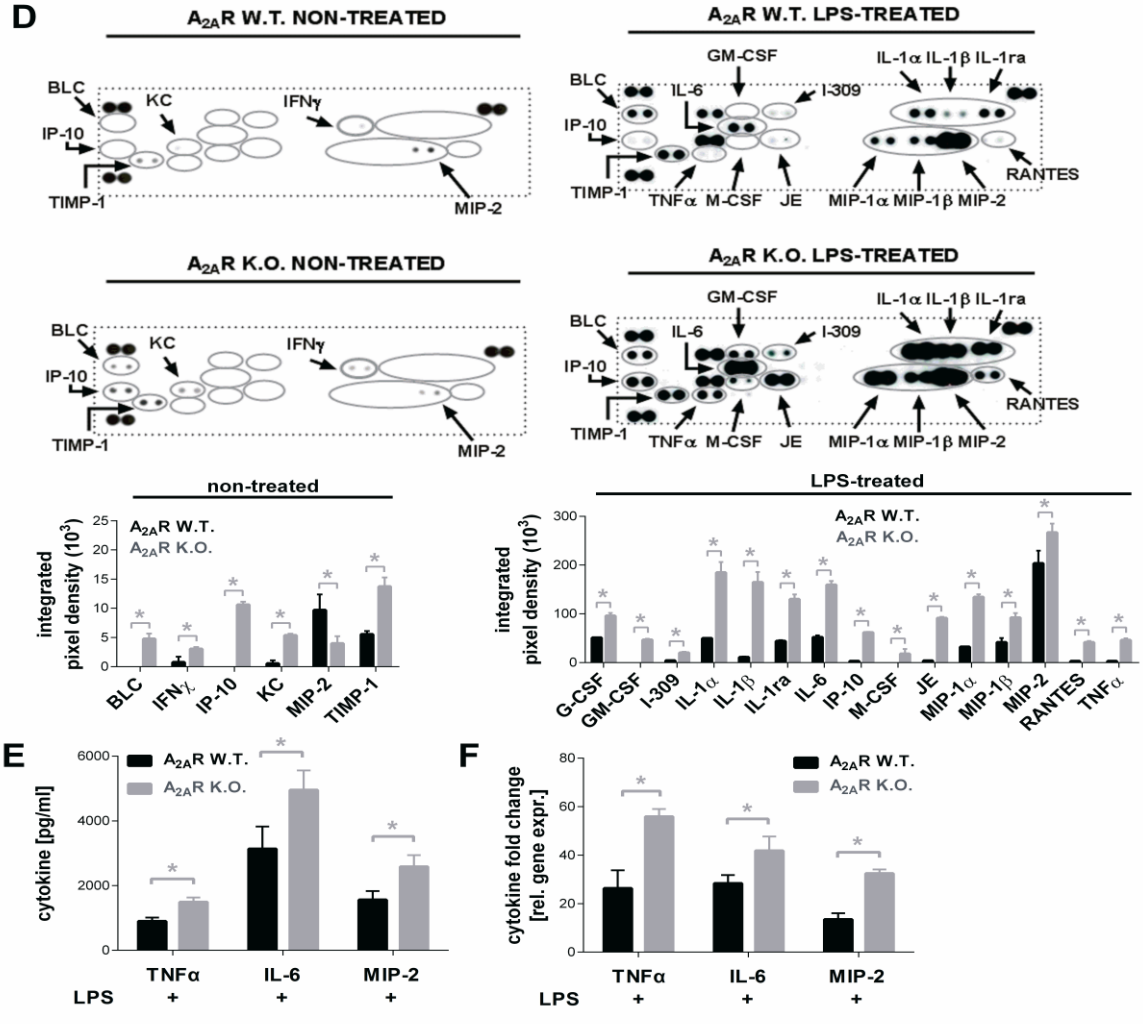

Fig. 1

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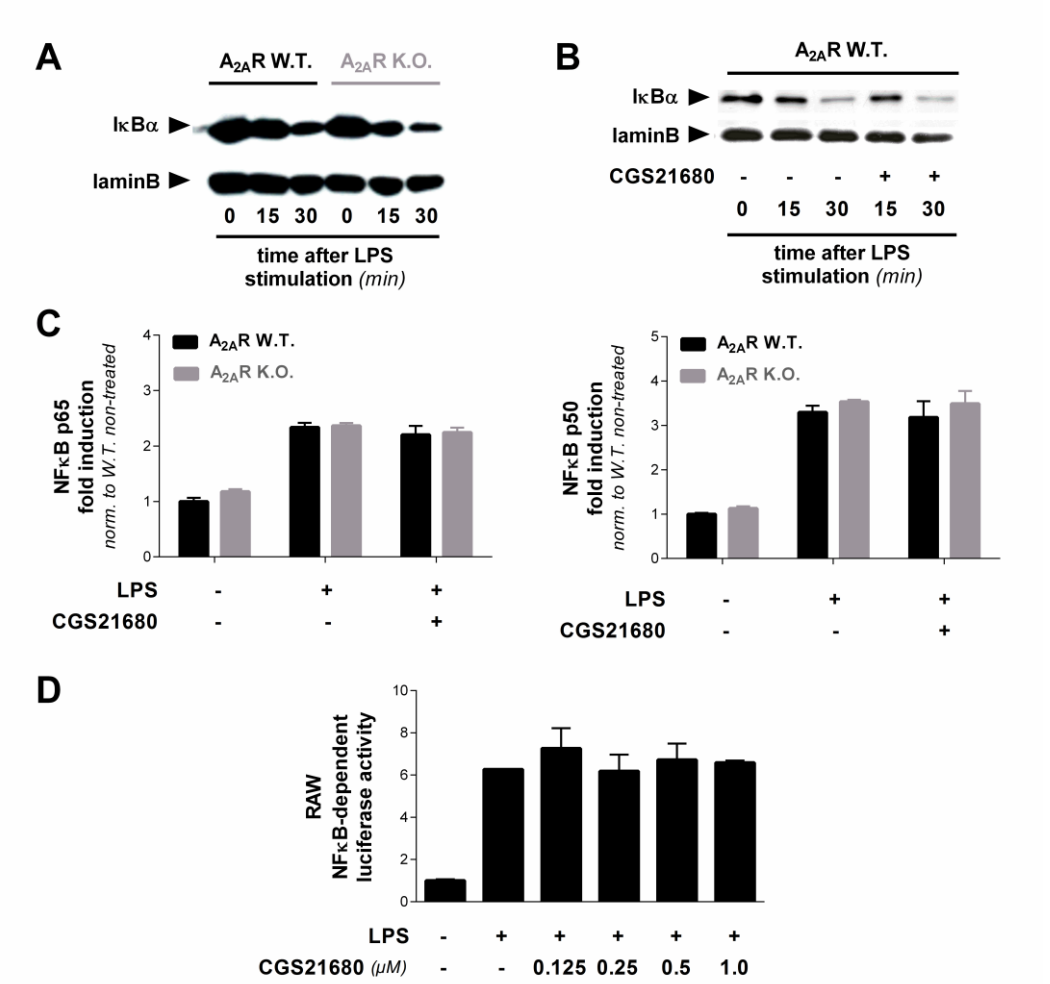

Fig. 2

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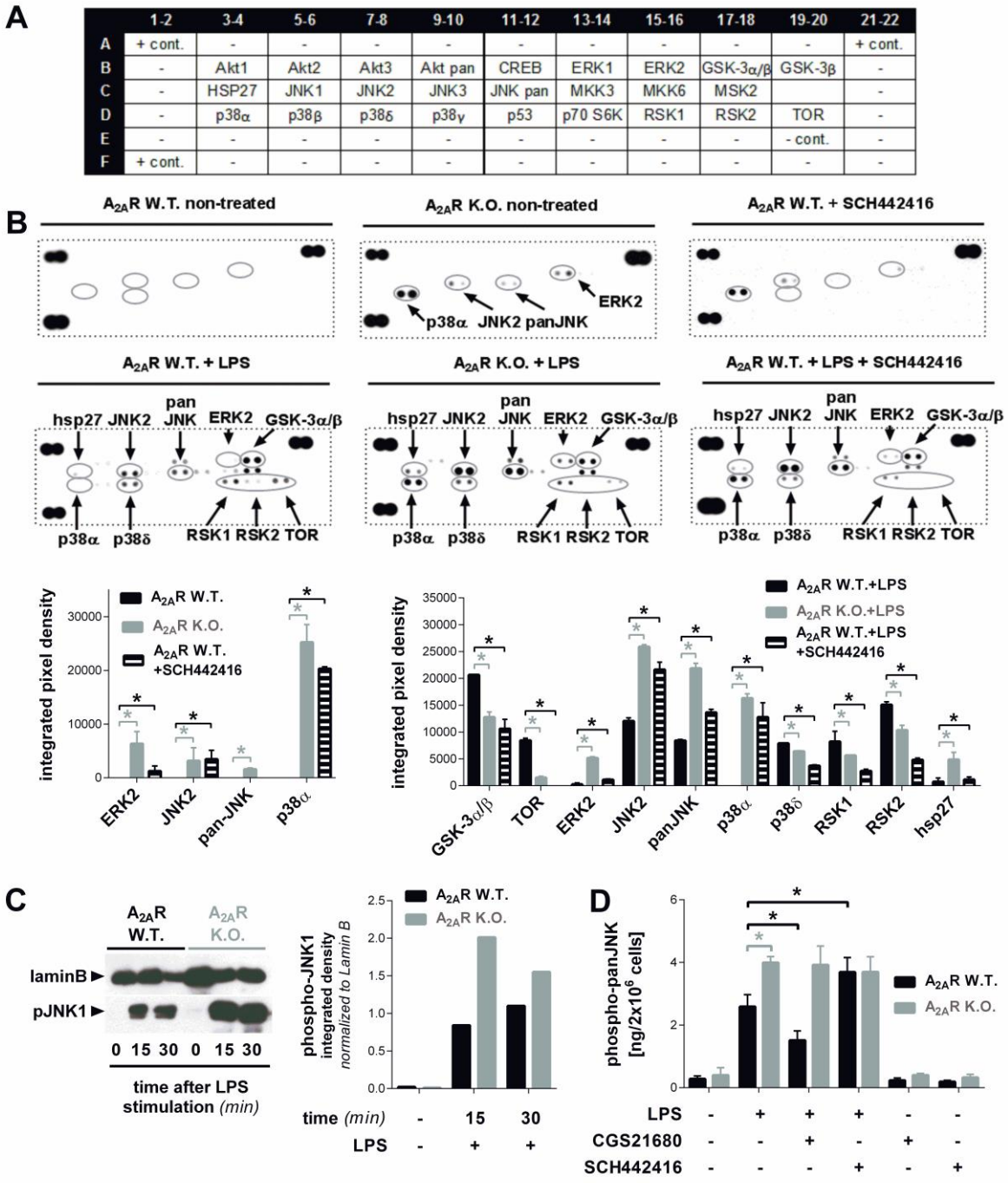
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A + cont. - - - - - - - - - + cont.
B - Akt1 Akt2 Akt3 Akt pan CREB ERK1 ERK2 GSK-3α/β GSK-3β -
C - HSP27 JNK1 JNK2 JNK3 JNK pan MKK3 MKK6 MSK2 -
D - p38α p38β p38δ p38γ p53 p70 S6K RSK1 RSK2 TOR -
E - - - - - - - - - - cont. -
F + cont. - - - - - - - - - -
B
A2AR W.T. non-treated
A2AR K.O. non-treated
A2AR W.T. + SCH442416
ERK2
p38α JNK2 panJNK
A2AR W.T. + LPS
A2AR K.O. + LPS
A2AR W.T. + LPS + SCH442416
hsp27 JNK2 pan JNK ERK2 GSK-3α/β
p38α p38δ RSK1 RSK2 TOR
integrated pixel density
A2AR W.T.
A2AR K.O.
A2AR W.T. +SCH442416
ERK2 JNK2 pan-JNK p38α
A2AR W.T.+LPS
A2AR K.O.+LPS
A2AR W.T.+LPS +SCH442416
GSK-3α/β TOR ERK2 JNK2 panJNK p38α p38δ RSK1 RSK2 hsp27
C
A2AR W.T.
A2AR K.O.
laminB
pJNK1
0 15 30 0 15 30
time after LPS stimulation (min)
phospho-JNK1 integrated density normalized to Lamin B
time (min) - 15 30
LPS - + +
D
phospho-panJNK [ng/2x10^6 cells]
LPS - + + + - -
CGS21680 - - + - + -
SCH442416 - - - + - +

Fig. 3

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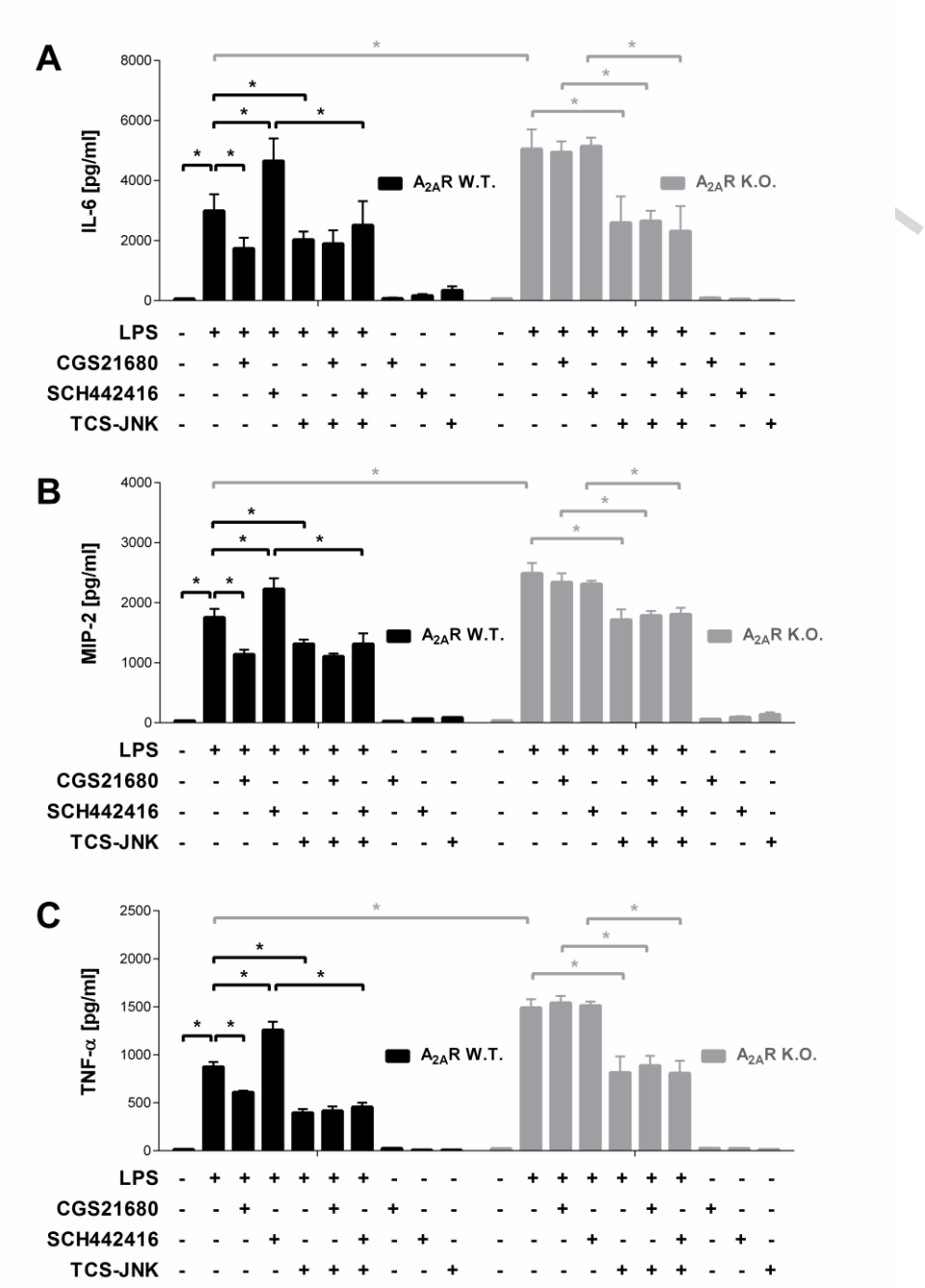

Fig. 4

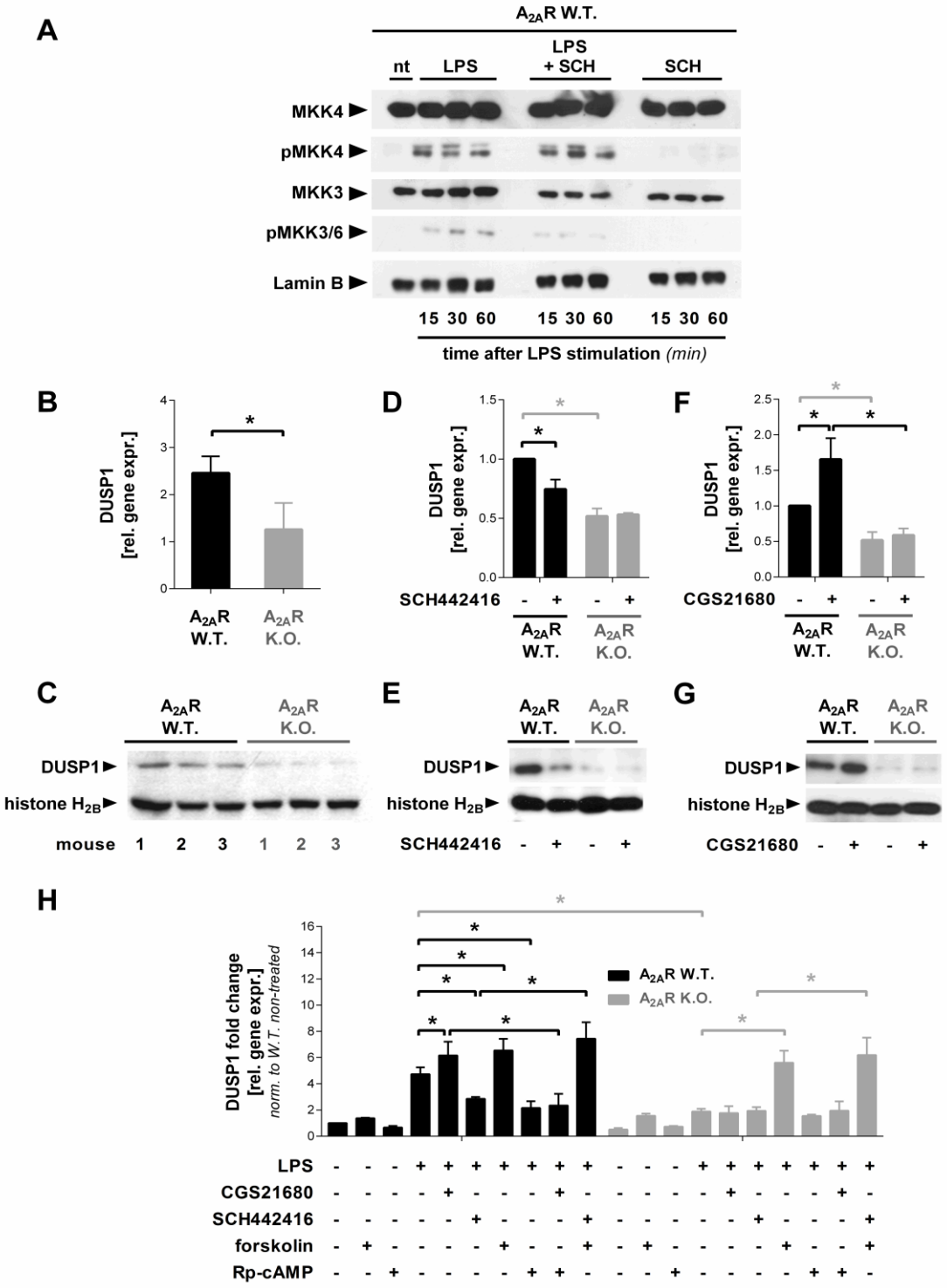
A
A2AR W.T.
nt
LPS
LPS + SCH
SCH
MKK4
pMKK4
MKK3
pMKK3/6
Lamin B
15 30 60 15 30 60 15 30 60
time after LPS stimulation (min)
B
DUSP1 [rel. gene expr.]
A2AR W.T.
A2AR K.O.
D
SCH442416
F
CGS21680
C
mouse 1 2 3 1 2 3
DUSP1
histone H2B
E
G
H
DUSP1 fold change [rel. gene expr.] norm. to W.T. non-treated
LPS
CGS21680
SCH442416
forskolin
Rp-cAMP

Fig. 5

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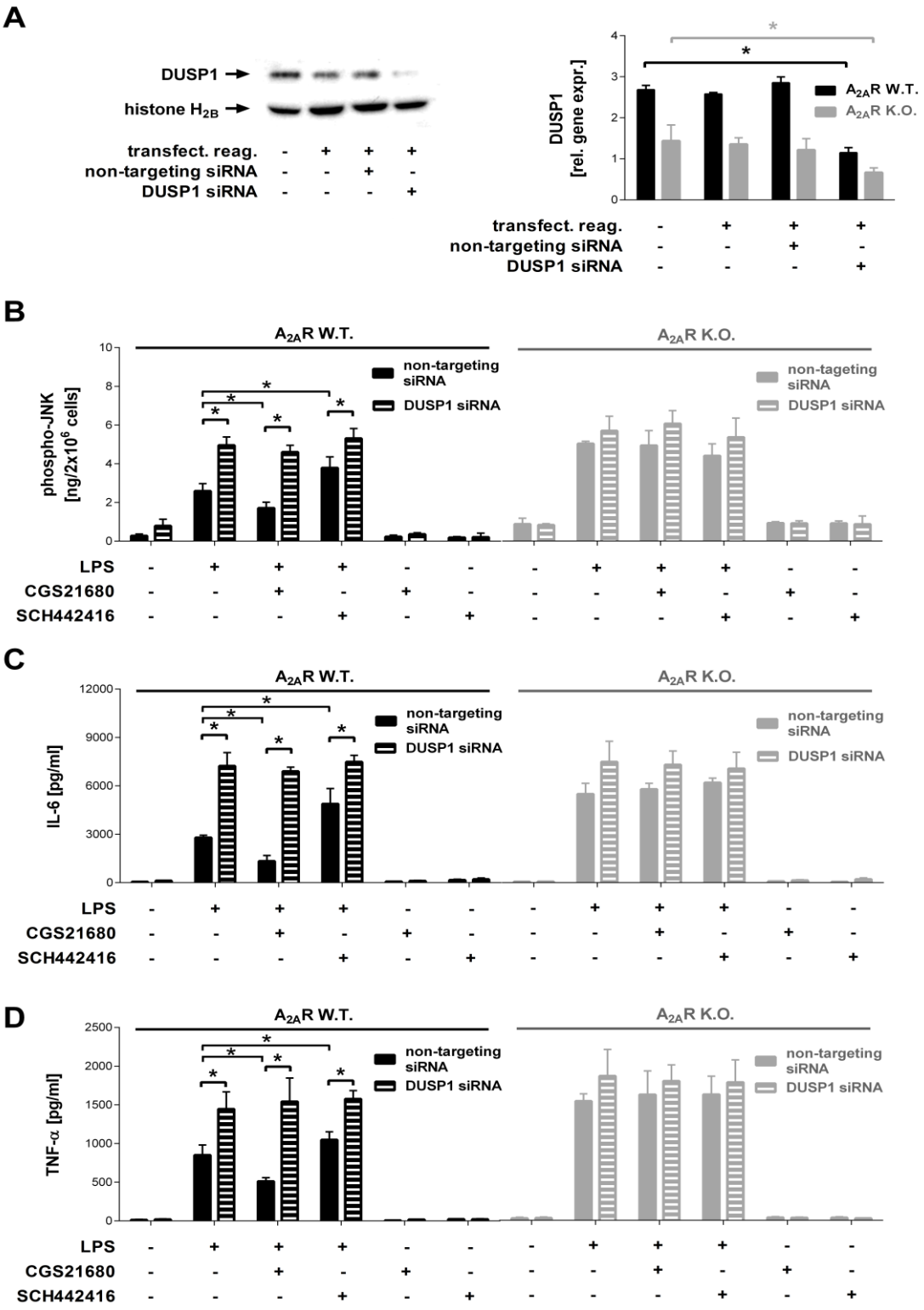

Fig. 6

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Highlights

- LPS stimulation of A2AR null macrophages results in a higher pro-inflammatory response
- Loss of A2ARs does not affect the LPS-induced NF-κB signaling pathway in macrophages
- Loss of A2ARs results in higher basal and LPS-induced MAPK phosphorylation
- A2AR signaling does not decrease MKK activation, but maintains high DUSP1 expression
- DUSP1 levels are regulated by A2ARs via the adenylate cyclase signaling pathway

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