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ORIGINAL ARTICLE

Transcriptomic effects of adenosine 2A receptor deletion in healthy and endotoxemic murine myocardium

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Abstract Influences of adenosine 2A receptor ($A_{2A}R$) activity on the cardiac transcriptome and genesis of endotoxemic myocarditis are unclear. We applied transcriptomic profiling (39 K Affymetrix arrays) to identify $A_{2A}R$-sensitive molecules, revealed by receptor knockout (KO), in healthy and endotoxemic hearts. Baseline cardiac function was unaltered and only 37 $A_{2A}R$-sensitive genes modified by $A_{2A}R$ KO (≥1.2-fold change, <5 % FDR); the five most induced are *Mtr*, *Ppbp*, *Chac1*, *Ctsk* and *Cnpy2* and the five most repressed are *Hp*, *Yipf4*, *Acta1*, *Cidec* and *Map3k2*. Few canonical paths were impacted, with altered *Gnb1*, *Prkar2b*, *Pde3b* and *Map3k2* (among others) implicating modified G protein/cAMP/PKA and cGMP/NOS signalling. Lipopolysaccharide (LPS; 20 mg/kg) challenge for 24 h modified >4100 transcripts in wild-type (WT) myocardium (≥1.5-fold change, FDR < 1 %); the most induced are *Lcn2* (+590); *Saa3* (+516); *Serpina3n* (+122); *Cxcl9* (+101) and *Cxcl1* (+89) and the most repressed are *Car3* (−38); *Adipoq* (−17); *Atgrl1/Aplnr* (−14); *H19* (−11) and *Itga8* (−8). Canonical responses centred on inflammation, immunity, cell death and remodelling, with pronounced amplification of toll-like receptor (TLR) and underlying JAK-STAT, NFκB and MAPK pathways, and a 'cardio-depressant' profile encompassing suppressed ß-adrenergic, PKA and Ca^{2+} signalling, electromechanical and mitochondrial function (and major shifts in transcripts impacting function/injury including *Lcn2*, *S100a8/S100a9*, *Icam1*/*Vcam* and *Nox2* induction, and *Adipoq*, *Igf1* and *Aplnr* repression). Endotoxemic responses were selectively modified by $A_{2A}R$ KO, supporting inflammatory suppression via $A_{2A}R$ sensitive shifts in regulators of NFκB and JAK-STAT signalling (IκBζ, IκBα, STAT1, CDKN1a and RRAS2) without impacting the cardio-depressant gene profile. Data indicate $A_{2A}Rs$ exert minor effects in un-stressed myocardium and selectively suppress NFκB and JAK-STAT signalling and cardiac injury without influencing cardiac depression in endotoxemia.

Keywords Adenosine · Adenosine 2A receptor · Anti-inflammatory · Cytokines · Endotoxemic myocarditis · Gene expression · Inflammation · Microarray · Sepsis

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Introduction

Uncontrolled inflammation with sepsis and the systemic inflammatory response syndrome induce multiple organ dysfunction, including cardiac abnormalities key to disease progression and mortality [1]. Unravelling the complex mechanisms governing

myocardial injury [1, 2] is not only fundamentally important but also reveals targets for manipulating outcomes. In this regard, adenosine 2A receptors ($A_{2A}Rs$) may fulfil a broadly suppressive role to limit inflammatory injury in multiple tissues [3–6] and enhance myocardial resistance to ischaemic/hypoxic insult, presenting a potentially useful therapeutic target [3, 7]. In heart, this G protein-coupled receptor (GPCR) influences coronary tone and angiogenesis, cardiac contractility, fibroblast growth and fibrosis and may mediate protection via ischaemic pre- and post-conditioning [8–10]. Inflammatory modulation contributes to this latter cardioprotection [9–11], together with the regulation of myocyte kinase signals to limit oxidative stress, mitochondrial dysfunction and cell death [12–14]. However, impacts of the $A_{2A}R$ on integrated myocardial responses to uncontrolled inflammation, and the mechanisms underlying such effects, remain to be elucidated.

While adenosine analogues and $A_{2A}R$ agonists can limit endotoxemic or septic injury in lung [15, 16], liver [17], brain [18] and heart [19–21], the roles of *intrinsic* $A_{2A}R$ activity are less clear. Receptor deletion fails to modify inflammatory markers/injury in some studies [22], reportedly worsens endotoxemic injury in heart [23] and lung [15, 24] and improves survival in models of polymicrobial sepsis [25, 26]. These divergent outcomes may reflect different cell- and organ-specific effects of $A_{2A}Rs$, for example promoting inflammasome formation and macrophage-dependent injury [27], impairing bacterial clearance [25], while activating cardiac survival signalling and suppressing inflammation [3, 7, 12–14]. Nonetheless, opposing effects of endogenous vs. exogenous (or amplified endogenous) adenosine are evident within cell types, for example inhibiting vs. promoting vascular myocyte NOS activation with inflammation [28]. The chronicity of receptor activation may also be critical to tissue-specific responses; while acute or transient increases in adenosine levels/receptor activity are generally beneficial (improving tissue perfusion, ischemic/hypoxic tolerance and reducing cytokines/inflammation), chronic elevations may be detrimental, exaggerating fibrotic processes via $A_{2B}Rs$ in lung [29] or A_{2A} and $A_{2B}Rs$ in liver [30, 31], for example. Whether acute vs. chronic $A_{2A}R$ activity induces opposing myocardial outcomes is unknown, though prolonged A_{2B} agonism limits rather than promotes fibrosis in injured myocardium [32], as does prolonged A_1 agonism [33].

Additional to questions regarding protective vs. deleterious effects of the $A_{2A}R$, the evolution of myocardial inflammatory injury and 'endotoxemic myocarditis' itself is complex and incompletely defined [1, 2]. Effects in non-cardiac tissues involve TLR/CD14-dependent NFκB and MAPK signalling and interferon/cytokine engagement of the JAK-STAT path [34–36]. These mechanisms likely participate in heart, with evidence NFκB and IκB kinase promotes cardiac dysfunction in sepsis/endotoxemia [37, 38], while JAK-STAT signalling mediates dysfunction and cell death in myocardial ischemia [39, 40]. In vivo observations suggest marked expansion of this signalling in intact myocardium [41, 42], contrasting in vitro evidence that cardiomyocyte NFκB and IκB kinase signalling is only transiently LPS responsive [43]. Importantly, these paths are inhibited by $A_{2A}Rs$ in other cells, with exogenous agonism decreasing [44, 45] and $A_{2A}R$ KO increasing NFκB activity [6]. Nonetheless, these paths can also promote myocardial stress-resistance and survival under conditions that include inflammatory cytokine challenge [46–48]. The molecular underpinnings of endotoxemic myocarditis in vivo warrant further investigation, as do the mechanisms countering this dysfunction (including $A_{2A}R$ activity). We thus undertook broad-scale transcriptomic profiling of hearts from WT and $A_{2A}R$ KO mice, at baseline and following endotoxin challenge: shifts in gene expression can shed light on both functions of the $A_{2A}R$, and pathogenesis and modulation of endotoxemic myocarditis. There are no prior analyses of transcriptome-wide effects of $A_{2A}R$ activity in myocardium, and relatively few of the in situ cardiac response to endotoxemia or sepsis [41, 42, 49].

Materials and methods

Animals

All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (publication number 85-25, revised 1996). This project (and protocols for care of animals) was approved by the Animal Care and Use Committees of St. Jude Children's Research Hospital and the University of Tennessee Health Sciences Center. The $A_{2A}R$ KO mice and WT littermates were obtained from a sub-colony of the original lines generated and characterized by Ledent et al. [50]. Analysis of cardiac tissue confirms ablation of $A_{2A}R$ transcript without compensatory shifts in other adenosine receptors (Fig. S1). Mice were bred and maintained on-site at St. Jude Children's Research Hospital, with standard laboratory food and water available ad libitum. Animal genotype was confirmed by PCR analysis of tail snips. All animals were originated from the same breeding series and were matched for age and weight. Male and female mice were used with equal representation in each experimental group. Both $A_{2A}R$ KO and WT littermate mice were randomly allocated to receive either an intraperitoneal injection of 20 mg/kg LPS isolated from *E. coli* (Sigma-Aldrich, St. Louis, MO) or an equal volume of sterile saline vehicle. Blood was sampled at 12 and 24 h of LPS challenge to assess shifts in blood cell counts and circulating cytokines/biomarkers, as detailed by us previously [23]. Mice were monitored for the development of symptoms of illness or distress (lethargy, piloerection, hunched posture and/or respiratory distress) during the initial 12 h post-injection and every 4 h thereafter; observation of

any combination of symptoms prompted immediate euthanasia. All efforts were made to minimize animal suffering and distress. No analgesic or anaesthetic was administered other than for euthanasia immediately on evidence of illness/distress.

We initially tested responses to 24 h LPS challenge in both young (14 week) and old (46–52 weeks) WT and $A_{2A}R$ KO mice. However, absent overt symptoms of illness/distress, there was nonetheless significant mortality in aged LPS-treated mice, as detailed in the "Results" section and in Supplementary material. Cause of death in older animals was not determined. As a result of mortality, detailed molecular interrogation of cardiac gene expression (n = 6–8 per group), together with analyses of cardiac function in isolated hearts (n = 8–9 per group) and haematological/biomarker assessment (n = 6–9 per group) is necessarily constrained to the young group of mice.

Langendorff perfusion and tissue sampling

After 24 h of LPS challenge, mice were anaesthetised with sodium pentobarbital (50 mg/kg intraperitoneally), a thoracotomy performed and hearts excised into ice-cold Krebs-Henseleit solution for Langendorff perfusion, as detailed previously [23], or sampling of ventricular tissue for RNA preparation. Briefly, for perfusions, the aorta was immediately cannulated and hearts perfused at 80 mmHg with modified Krebs-Henseleit solution containing (in mM): NaCl, 120; NaHCO3, 25; KCl, 4.7; CaCl2, 2.5; MgCl2, 1.2; KH2PO4, 1.2; D-glucose, 15 and EDTA, 0.5. Perfusion fluid was maintained at 37 °C and bubbled with a mix of 95 % O2/5 % CO2 at 37 °C to provide a pH of 7.4. Ventricular function was monitored via a fluid-filled plastic film balloon in the left ventricle, connected to a P23 XL pressure transducer (Viggo-Spectramed, Oxnard, CA, USA). Coronary flow was monitored via a flow-probe in the aortic perfusion line, connected to a T206 flowmeter (Transonic Systems Inc., Ithaca, NY, USA). Functional data were recorded at 1 KHz on MacLab data acquisition system (ADInstruments, Castle Hill, Australia). Statistical comparisons of cardiovascular (Table 1) and blood parameters (Figs. 1 and 2) between groups were made via analysis of variance (ANOVA), with a Newman-Keuls post hoc test for specific comparisons. A $P < 0.05$ was considered indicative of significance in all tests.

RNA preparation and microarray hybridisation

Ventricular tissue was isolated, frozen in liquid N_2 and stored at −80 °C until analysis. Tissue was subsequently homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA isolated according to manufacturer's protocol. The RNA was further treated with 50 U of DNase I (Promega, Madison, WI) for 15 min at 37 °C and purified on RNeasy spin columns (Qiagen, Hilden, Germany). Total RNA yield and integrity were determined spectrophotometrically and via capillary electrophoresis on a 2100 BioAnalyzer (Agilent, Palo Alto, CA), respectively.

Microarray experiments were performed at the St. Jude Hartwell Center Core Facility according to manufacturers' protocols. Briefly, first- and second-strand complementary DNA (cDNA) synthesis reactions were performed using the SuperScript Choice System (Invitrogen, Carlsbad, CA) followed by in vitro transcription using biotin-labelled dNTPs (ENZO Diagnostics, Farmingdale, NY). Complementary RNA (cRNA) samples were fragmented and individually hybridized to GeneChip® Mouse Genome 430 2.0 microarrays (Affymetrix, Santa Clara, CA). Each microarray quantified expression of over 39,000 transcripts, including full-length mRNA sequences and expressed sequence tags (ESTs). Following hybridization, microarrays were washed and stained with streptavidin–phycoerythrin (Molecular Probes, Eugene, OR) before scanning. Image files were converted into probe-set data (*.CEL files) via Affymetrix MAS 5.0 software. Experimental data are accessible through GEO Series accession number GSE44363 at http://www.ncbi.nlm.nih.gov/geo.

Microarray data and statistical analyses

Raw expression data were background corrected, normalized and $\log_2$ transformed using the Robust Multichip Average (RMA) method in BioConductor/R [51]. Data were filtered to include only transcripts detected on ≥3 arrays, with 25,646 transcripts passing this quality criterion (i.e. consistently detected in cardiac tissue). Log_2 ratio values were generated by subtracting median expression values for WT vehicle-treated animals from each sample per probe before importing into TIGR MeV 4.0 software for statistical analysis [52]. The Significant Analysis of Microarrays (SAM) algorithm was used to correct for multiple comparisons and non-parametrically select differentially expressed genes using a median false discovery rate (FDR) ≤1.0 % [53]. In addition, a two-factor analysis of variance (ANOVA) was performed. To generate a data sets similar in size and false-positive rates, a P value <0.005 was employed. The lists of differentially expressed genes generated via SAM or ANOVA were then compared to identify 8110 shared transcripts for further investigation and validation (Fig. S2). This output was subjected to appropriate pair-wise SAM comparisons, incorporating a 1.5-fold change cut-off [54]. Significant transcripts were annotated using Ingenuity Pathway Analysis (IPA) (v8.7; Ingenuity® Systems, Redwood City, CA, USA), providing insight into biological/molecular themes over-represented in response to $A_{2A}R$ deletion and/or LPS. In brief, for each pathway/process,

Table 1 Ex vivo cardiovascular function in perfused for hearts from WT and A_{2A}R KO mice

Functional parameter	WT (n = 8)	A_{2A}R KO (n = 8)	WT + LPS (n = 9)	A_{2A}R KO + LPS(n = 9)
Coronary flow (ml/min/g)	16.7 ± 0.7	15.6 ± 1.2	16.9 ± 1.7	15.9 ± 0.6
Heart rate (beats/min)	341 ± 7	341 ± 7	352 ± 9	353 ± 9
Systolic pressure (mmHg)	113 ± 6	118 ± 5	80 ± 7*	86 ± 3*
+dP/dt (mmHg/s)	6039 ± 247	6039 ± 163	4310 ± 422*	4489 ± 220*
−dP/dt (mmHg/s)	−3664 ± 129	−3718 ± 132	2666 ± 197*	−2846 ± 105*
Coronary 'Supply:Demand' (ml/min/g/mmHg)	0.15 ± 0.01	0.13 ± 0.02	0.21 ± 0.02*	0.18 ± 0.02*

All data are means ± S.E.M. Functional parameters were measured after 30 min of normothermic aerobic perfusion. Intrinsic heart rate is recorded immediately prior to pacing. *P < 0.05 vs. corresponding values in Vehicle-treated hearts. Coronary 'Supply:Demand' was calculated as the ratio of coronary flow rate (O_2 supply) relative to ventricular systolic pressure (reflecting myocardial O_2 demand). dP/dt, first derivative of pressure over time. *P < 0.05 vs. untreated. A_{2A}R KO did not independently modify functional parameters

the fraction of differentially expressed genes was compared with the fraction of total genes in that path (shown in tables as the 'Ratio', useful for determining which pathways/processes overlap the most with altered genes in specific datasets). The probability of involvement of the respective number of modified transcripts in a path/process is expressed as a P value or range (values <0.05 considered significant).

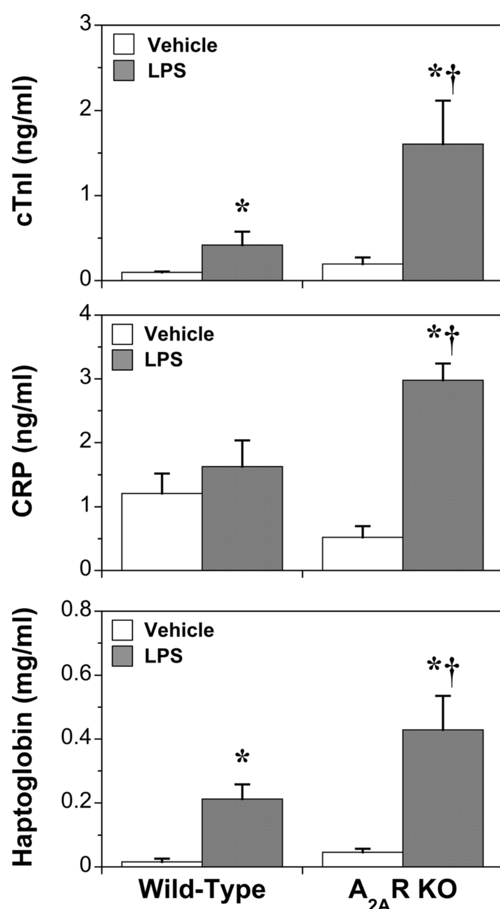


Fig. 1 Effects of A_{2A}R KO and LPS (24 h) on markers of cardiac damage (TnI) and systemic inflammation (CRP) and the acute phase response (haptoglobin). Data represent means ± S.E.M. *P < 0.05 vs. vehicle; †P < 0.05 vs. wild-type

Validation of expression changes and *Adora* expression via RT-qPCR

Two-step RT-qPCR, utilizing SYBR Green I, was employed to confirm differential gene expression for 11 transcripts (primer details provided in Table S1), as previously described [54]. Six additional genes (*Actb*, *Gapdh*, *Hprt1*, *Pgk1*, *Ppia and Ubc*) were assessed using GeNorm to determine utility as reference genes [55]. Following GeNorm assessment, *Actb* was found to be most stable (M = 0.03) and served as the endogenous reference control for all messenger RNAs (mRNAs) assessed via RT-qPCR. Briefly, 1 μg total RNA was used to synthesize cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RT-qPCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Final reaction volumes (10 μL) included 5 μL iQ SYBR-Green Supermix (Bio-Rad, Hercules, CA), 100 nM of each primer and 4 μL of a 1:20 dilution of cDNA. Optimal qPCR cycling conditions entailed an initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s/62 °C for 60 s. After the final PCR cycle, reactions underwent melt curve analysis to detect non-specific amplicons. All reactions were performed in triplicate, with each plate containing an equal number of samples from each group, a calibrator control derived from a pool of all cDNA samples and a no-template control. PCR amplification efficiencies (90–110 %) for each primer pair were calculated using a 5-log serial dilution of calibrator sample. PCR data were

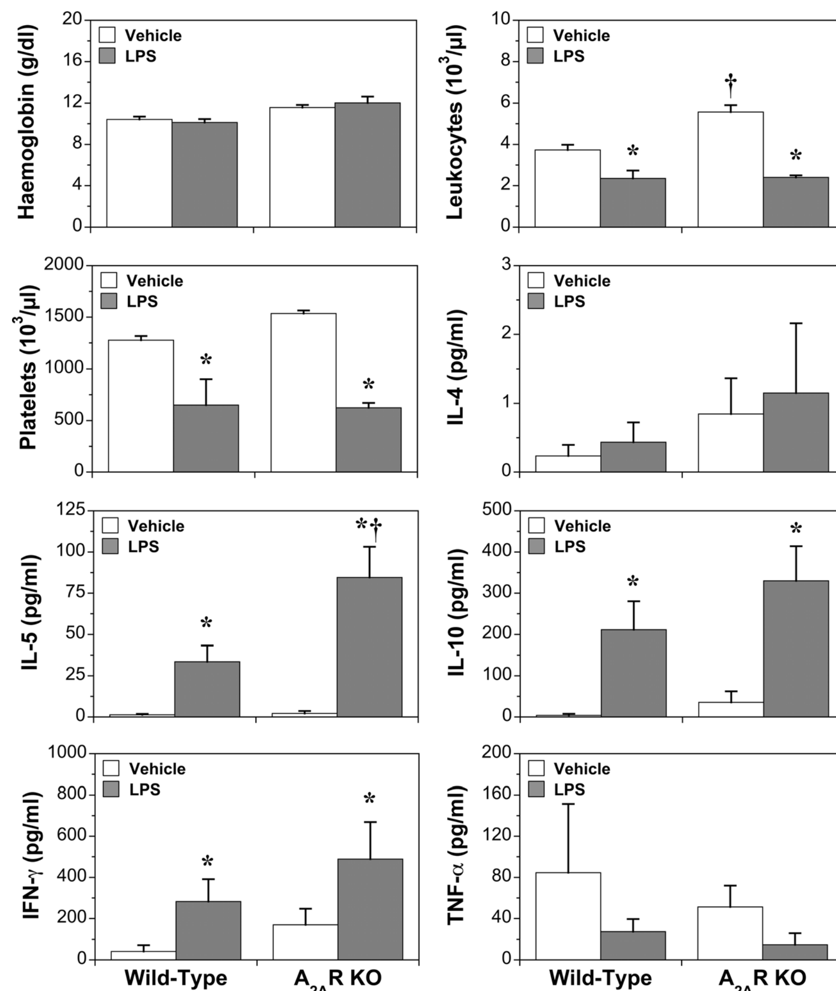


Fig. 2 Effects of A_{2A}R KO and LPS (24 h) on haematological parameters and cytokines. Data represent means ± S.E.M. *$P < 0.05$ vs. vehicle; †$P < 0.05$ vs. wild-type

analysed using CFX Manager v1.6 (Bio-Rad, Hercules, CA). Baseline subtractions and threshold settings above background were applied to all data. The calibrator sample was used to normalize inter-assay variations, with the threshold coefficient of variance for intra- and inter-assay replicates <1 % and <5 %, respectively. Normalized expression ($\Delta\Delta C_q$) was calculated, with mRNAs normalized to *Actb* levels and the calibrator control then $\log_2$-transformed.

Results

Impact of A_{2A}AR deletion on cardiovascular function and inflammatory markers

Deletion of the A_{2A}R was confirmed in cardiac tissue, with no compensatory changes in transcription of the other three sub-types (Fig. S1). Receptor deletion did not modify cardiac or coronary function in healthy or endotoxemic hearts (Table 1). Cardiovascular, blood cell and cytokine responses to LPS in WT and A_{2A}R KO mice have been reported by us previously [23]. Similar outcomes were apparent here for markers of inflammation and injury (Fig. 1), haematological parameters and cytokines (Fig. 2) and cardiac function (Table 1). Data confirm significant inflammatory activation, cellular injury and cardiac dysfunction with LPS, with A_{2A}R deletion amplifying changes in IL-5 and markers of cardiac injury (TnI) and systemic inflammatory stress (haptoglobin, CRP), without altering myocardial dysfunction or circulating levels of IL-4, IL-10, IFNγ or TNFα.

Impact of A_{2A}AR deletion on survival

Interestingly, we acquire preliminary evidence LPS-dependent mortality is age- and sex-dependent and selectively exaggerated by A_{2A}R KO in older males (Fig. S3). Initially,

testing responses to LPS challenge in young (14 week) and middle-aged (46–52 week) mice—since age may exaggerate impacts of endotoxemia [56]—we recorded 20 % mortality in older WT mice with A_{2A}R KO specifically reducing survival to <20 % in older males (Fig. S3). No mortality was recorded in young mice challenged with LPS (or any vehicle-treated groups). There is thus a trend to greater mortality with age, with a major survival effect of A_{2A}R activity in older males. While molecular interrogations here are thus limited to young mice (in which LPS was non-lethal), it is possible A_{2A}R activity may be increasingly crucial to survival in older, stress-intolerant males (whereas younger animals and females may possess intrinsically greater resistance to injury/death [57]). This is discussed further in the online Supplementary material.

Cardiac transcriptomic response to A_{2A}R KO

Few transcriptional differences were detected between WT and A_{2A}R KO hearts, with the amplitude of changes modest (up to threefold). Employing an initial 1.5-fold cut-off and 1 % FDR identified only 13 genes (Table 2). To enhance the power of subsequent pathway analysis, these criteria were relaxed to 1.2-fold and 5 %, encompassing 37 altered transcripts (Table 2). There was a little impact of A_{2A}R deletion on inflammatory mediators, with modest induction of a CXC chemokine (*Ppbp*, +2.1), a regulator of cell migration/adhesion and cytokinesis (*Iqgap1*, +1.6) and a hemopoietic cytokine (*Tslp*, +1.6), together with repression of *Hp* (−2.7) and an Ig adhesion molecule regulating T-lymphocyte development (*Mpzl2*, −1.7) (Table 2).

Analysis via the IPA suite identified 112 canonical pathways sensitive to A_{2A}R activity in healthy hearts (Table S2), the most highly modified shown in Table 3. The top five included relaxin signalling, cardiac β-adrenergic signalling, cellular effects of sildenafil, PKA signalling and photo-transduction. The influence of A_{2A}R KO on these and other paths can be attributed to repression of *Prkar2b* (−1.8), *Gnb1* (−1.7) and *Pde3b* (−1.7), collectively impacting G protein-coupled cAMP/PKA dependent signalling (encompassing relaxin, α- and β-adrenergic, NO, Ca^{2+} and hypertrophic signalling). Additionally, *Map3k2* (−2) and *Nfat2c* (+1.3) span many paths modified by A_{2A}R KO. Modulation of these five transcripts contributes to 48 of the top 50 A_{2A}R-sensitive canonical pathways. Overall, A_{2A}R deletion exerts an inhibitory effect on G protein-coupled, cAMP/PKA, and MEKK2 signalling downstream of this and other GPCRs.

Multiple biological functions appear sensitive to A_{2A}R KO (see Table S3), the most significant (Table 4) revolving around cellular development, growth, movement and death, together with humoral immunity and haematological development and intercellular signalling. Toxicological functions included high representation of liver processes (fibrosis, damage, steatosis, hepatitis and inflammation) together with six cardiac functions (Table 4). The latter included cardiac arteriopathy (*Ank3*, *Pon1*, *Cux2*, *Gk5*, *Pde3b*, *Dgat1* and *Nsmce1*), infarction (*Pon1* and *Acta1*) and transcripts involved in failure and hypertrophy (*Pde3b* and *Nfat2c*).

Cardiac transcriptomic response to LPS

Analysis via the SAM algorithm (1.5-fold threshold, 1 % FDR) identified 4146 transcripts modified after 24 h of LPS challenge (see Table S4 for full details). Figure 3 presents the 25 most induced and repressed transcripts, with responses in A_{2A}R KO hearts shown for comparison. Many of the most highly modified are predictably involved in inflammation/innate immunity, together with tissue development/remodelling. The most LPS-responsive cytokines/chemokines are shown in Fig. 4, with responses in KO hearts highlighted.

Functional classifications, using a twofold threshold to narrow the focus to the most highly modified paths, identify 236 canonical pathways (Table S5**)**, with the most significantly modified presented in Table 5. Data confirm a profound inflammatory and immune response, with marked upregulation of TLR/MyD88 and interferon signalling (Fig. 5), the acute phase response (Fig. S4), IRF activation (Fig. S5) and PRR- and RIG-1-like signalling (Figs. S6 and S7). These changes primarily reflect shifts in underpinning NFκB, JAK-STAT, MAPK and PI3K/Akt signalling (Figs. S8–S12). Additionally, LPS upregulated cell death signalling (Figs. S13 and S14) and modified paths involved in cellular differentiation, movement, growth and remodelling (e.g. Figs. S15 and S17). The top LPS-sensitive biological functions are summarized in Table S6, with biologic and cardiovascular toxicological responses fully detailed in Table S7 and S8 in the Supplementary material.

Unsurprisingly, many highly responsive transcripts were involved in inflammation (Figs. 3 and 4, Table 5): within the most induced are interferon-related genes (*Ifit1*, *Ifit2*, *Ifit3*, *Igtp1*, *Igtp2*, *Igtp3*, *Iigp1*, *Iigp2*, *Ifi44* and *Irf7*) and inflammatory/immune modulators (*Lcn2*, *Saa3*, *Socs3*, *S100a8*, *S100a9*, *Mpa2l*, *Cxcl1*, *Cxcl9*, *Cxcl10*, *IL6*, *Ptx3*, *Serpina3n*, *Csf3* and *Mt2*). Several are potentially injurious to the heart: the most highly induced transcript encodes lipocalin-2, which regulates inflammation and matrix degradation, is implicated in heart failure [58] and promotes cardiac apoptosis [59]; CXCL1 is a potent neutrophil chemoattractant; CXCL9 stimulates cytokine production and T-cell proliferation/recruitment; CXCL10 mediates $CXCR3^{+}$ cell migration and augments inflammation. Conversely, some transcripts encode anti-inflammatory and potentially protective molecules: SERPINA3N (α1-antichymotrypsin) inhibits proteases involved in inflammation; the pentraxin PTX3 is expressed in heart with inflammation and limits cell death; SOCS3 is a negative feedback regulator of IL-6 signalling, implicated in sex-dependent cardiac stress-resistance;

Table 2 Genes modified by $A_{2A}R$ KO in healthy myocardium

Gene	Gene name	Affymetrix ID	Fold-Change	%FDR
	UpRegulated			
Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase	1439811_at	2.83	0.00
Ppbp	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	1418480_at	2.08	3.93
Chac1	ChaC, cation transport regulator homologue 1 (*E. coli*)	1451382_at	1.85	0.00
Ctsk	cathepsin K	1450652_at	1.84	0.00
Cnpy2	canopy 2 homologue (zebrafish)	1416507_at	1.67	0.00
Iqgap1	IQ motif containing GTPase activating protein 1	1445724_at	1.64	3.85
Tslp	thymic stromal lymphopoietin	1450004_at	1.63	3.93
Nav3	neuron navigator 3	1456144_at	1.61	0.00
Cux2	cut-like homeobox 2	1447500_at	1.60	0.00
Slc38a1	solute carrier family 38, member 1	1454764_s_at	1.47	0.00
Nsmce1	non-SMC element 1 homologue (*S. cerevisiae*)	1436121_a_at	1.42	0.00
Gk5	glycerol kinase 5 (putative)	1436210_at	1.38	3.93
Rpl22	ribosomal protein L22	1448398_s_at	1.37	0.00
C11orf75	chromosome 11 open reading frame 75	1419403_at	1.37	0.00
Slco5a1	solute carrier organic anion transporter family, member 5 A1	1440874_at	1.31	4.21
Nfatc2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	1426032_at	1.28	4.70
	Downregulated			
Dgat1	diacylglycerol O-acyltransferase homologue 1 (mouse)	1418295_s_at	−1.24	2.39
Ticam1	toll-like receptor adaptor molecule 1	1454676_s_at	−1.24	3.05
Mrap	melanocortin 2 receptor accessory protein	1451371_at	−1.31	3.05
Ppa1	pyrophosphatase (inorganic) 1	1416939_at	−1.32	4.31
Ell2	elongation factor, RNA polymerase II, 2	1450744_at	−1.33	2.53
Fmod	fibromodulin	1456084_x_at	−1.47	4.31
Ptger3	prostaglandin E receptor 3 (subtype EP3)	1450344_a_at	−1.49	2.68
Pkdcc	protein kinase domain containing, cytoplasmic homologue (mouse)	1454838_s_at	−1.50	4.61
Ank3	ankyrin 3, node of Ranvier (ankyrin G)	1457288_at	−1.58	3.05
Sacs	spastic ataxia of Charlevoix-Saguenay (sacsin)	1434958_at	−1.61	2.39
Pon1	paraoxonase 1	1418190_at	−1.68	0.00
Gnb1	guanine nucleotide binding protein (G protein), beta polypeptide 1	1425908_at	−1.72	4.54
Mpzl2	myelin protein zero-like 2	1416236_a_at	−1.74	0.00
Pde3b	phosphodiesterase 3B, cGMP-inhibited	1433694_at	−1.74	0.00
Prkar2b	protein kinase, cAMP-dependent, regulatory, type II, beta	1438664_at	−1.78	3.05
Slc16a9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	1429726_at	−1.84	0.00
Map3k2	mitogen-activated protein kinase kinase kinase 2 (MEKK2)	1438719_at	−2.01	0.00
Cidec	cell death-inducing DFFA-like effector c	1452260_at	−2.08	0.00
Acta1	actin, alpha 1, skeletal muscle	1427735_a_at	−2.14	2.68
Yipf4	Yip1 domain family, member 4	1426417_at	−2.51	4.54
Hp	haptoglobin	1448881_at	−2.70	0.00

Data shown for transcripts for which myocardial expression levels were modified by $A_{2A}R$ KO by a factor of ≥1.2-fold (FDR <5 %)

metallothionein-2, induced by STAT3-related signalling, mediates cardioprotection and can limit dysfunction in sepsis.

Cardio-depressant molecular profile Major pathways critical to contractile function were substantially impacted, identifying a ‘cardio-depressed’ molecular profile in hearts of endotoxemic mice (Figs. S18–S21). β-Adrenergic signalling was broadly suppressed (Fig. S18), including reduced transcripts for β_1-adrenergic receptor, G protein, adenylate cyclase, PKA and related signalling elements (*Ppp1r14c*, *Gng11*, *Prkar2b*, *Pde7a*, *Ppp1r3c*, *Ppp1r14a*, *Pkia* and *Adcy7*), together with induction of *Pde4b* (AMP-dependent phosphodiesterase). The PKA pathway itself was substantially suppressed

(Fig. S19), including reductions in PKA RIα (*Prkar1a*), PKA RIIα (*Prkar2a*), PKA RIIβ (*Prkar2b*), PKAα (*Prkaca*) and PKA-Cβ (*Prkacb*), while PKA RIβ (*Prkar1b*) was modestly induced (+1.5). Transcripts for $G_{\alpha s}$ and $G_{\alpha q}$ also declined.

Cellular Ca^{2+} signalling paths were repressed (Fig. S20), with reductions in key elements including: *Camk1d*, *Ryr3*, *Casq1*, *Mef2c*, *Casq2*, *Calm1*, *Hdac9*, *Myh7b*, *Tpm2*, *Myh11*, *Rcan1*, *Myl7*, *Atp2b2*, *Prkar2b*, *Tpm3* and *Asph*. Mitochondrial dysfunction (Fig. S21) is also evidenced by repression of complex I/NADH dehydrogenase components (including *Ndufv2* and *3*; *Ndufs4*, *5*, *6*, *7* and *8*; *Ndufa3*, *4*, *5*, *6* and *8*; *Ndufab1*, *Ndufaf1*, *Ndufb2*, *3*, *4*, *7*, *9*, *10* and *11*), together with Complex II components (including *Sdhc*, *Sdhd*, *Uqcrb*, *Uqcrc1* and *Uqcrfs1*). Uncoupling proteins *Ucp2* and *Ucp3* were also induced, while *Ucp1* was repressed. Transcript for the major activator of mitochondrial biogenesis (*Ppargc1a*) was repressed.

Adenosine-related transcripts There were limited impacts on the adenosinergic system itself, with two- to threefold upregulation of adenosine deaminase, 1.5-fold repression of adenosine kinase, 1.7-fold induction of S-adenosylhomocysteine hydrolase and twofold induction of the ecto-nucleotidase CD39. These changes may modify patterns of cellular adenosine generation vs. uptake and metabolism, potentially altering receptor activation in endotoxemic tissue. A small 1.3-fold rise in *Adora2b* expression was also detected.

Impact of A_{2A}R KO on transcriptomic responses to endotoxemia

Interestingly, A_{2A}R activity does not broadly suppress the effects of endotoxemia on myocardial gene expression, with 90 % of transcriptomic responses unaltered by receptor KO. For example, Fig. 3 presents response profiles for the 25 most induced or repressed transcripts in WT hearts, with most shown to be insensitive to A_{2A}R KO. Deletion selectively enhanced induction of *Lcn2*, *Igtp*, *Cxcl5*, *S100a8*, *Iigp2*, *Cxcl2* and *Iigp1* and repression of *C1qtnf9*, *Colec11* and *Inmt*, while reducing induction of *Ifit3* and repression of *Adipoq*, *Gpr22*, and *Scube2*, *Ifit3* (Fig. 3). These effects support A_{2A}R modulation of inflammatory and interferon-related signalling responses. Nonetheless, the pattern of LPS-dependent cytokine/chemokine change was largely insensitive to A_{2A}R KO, with specific enhancement of *Cxcl5*, *Cxcl2*, *Il6* and *Csf3* induction (Fig. 4). Figure 6 summarizes the select effects of A_{2A}R activity (revealed via KO) on TLR- and interferon-triggered NFκB and JAK-STAT signalling responses to LPS (generally repressing these pathways).

To gain further insight into these specific effects of A_{2A}R KO, we identified those LPS-responsive transcripts whose expression changes were increased or reduced ≥1.5-fold by A_{2A}R KO (Table S9). Effects of LPS on 282 genes were augmented ≥1.5-fold following A_{2A}R KO, with a further 9 responsive to LPS specifically in KO and not WT hearts (Table S9). The most highly augmented included 25 transcripts increased ≥twofold by KO. Many A_{2A}R-sensitive transcripts are associated with pro-inflammatory signalling (e.g. *Igtp*, *Lcn2*, *Cxcl5*, *S100a8*, *Iigp2*, *Cxcl2*, *Il6*, *Ifi202b*, *Hp* and *Cxcl9*), with data supporting inhibitory effects of the A_{2A}R on the responsiveness of canonical pathways that include the acute phase response, glucocorticoid receptor, Erk/MAPK, HIF1α and JAK-STAT signalling (Table 6).

Deletion of the A_{2A}R reduced or entirely eliminated 134 gene responses to LPS (Table S9). Responses entirely A_{2A}R-dependent included repression of *Sacs*, *Cidec*, *Gnb1l*, *Mtap1b*, *Msi2*, *Cdh2*, *Lpgat1*, *Tbx3*, *Slc38a1*, *Sdc1*, *Reln*, *Ext1* and *Pvrl3*, and induction of *Rit1, Ezh1, Eif4ebp1* and *Abhd4* (Table S9). The transcripts most sensitive to A_{2A}R KO included *Dnmt3a*, *Qk*, *Sema5a*, *Srpk2*, *Car3*, *Ucp1*, *Rsad2* and *Dnajb14*. Highly LPS-responsive changes counteracted by A_{2A}R KO included: *Ifit3*, *Rsad2*, *Cxcl13*, *Ms4a6d*, *Oasl1*, *Ms4a4c*, *Sema5a*, *Tyki* and *Hmox1*. Intrinsic A_{2A}R activity thus also promotes LPS-dependent shifts in select genes associated with immune/inflammatory processes, including orphan nuclear receptor and PPARα activation, PRR responses to infection, AMPK signalling and NFκB signalling (Table 6).

In terms of biological functions, A_{2A}R activity reduced LPS responsiveness of paths related to cellular movement, growth, signalling and death, immune cell trafficking and haematological and cardiovascular development (Tables 7 and S10). Conversely, A_{2A}R activity appears to augment responses in pathways related to metabolism and transport, together with organ, haematological, cardiovascular and lymphoid development (Table 7), with complete details of pathway responses modified by A_{2A}R KO included in Table S10. Deletion of the A_{2A}R also influenced multiple toxicological functions; of 35 responses countered by A_{2A}R KO, 12 were cardiac-specific categories (Table S11), with reduced *Adipoq* and *Hmox1* responses involved in ~half. Of 46 toxicological function responses enhanced by A_{2A}R KO, a third was cardiac categories. Enhanced responses for *Serpine1*, *Kckn3* and *Ppargc1a* are involved in a majority of these and are also implicated in cardiac dysfunction.

Importantly, a relatively small set of A_{2A}R responsive genes are implicated in a majority of these A_{2A}R sensitive biological pathways and processes and can be considered A_{2A}R-dependent 'nodes' or points of regulatory convergence. Of the top 50 pathway responses *augmented* by A_{2A}R KO, *Pi3kr1* is implicated in 42; *Rras2* in 40; *Stat1, Vegfc, Pgf* and *Pak6* each in 12; *Atf4* in 11; *Cdkn1a* in 9 and *Rhoj* and *IL6* each in 8 (Tables 6 and S10). Of the leading 50 canonical responses *inhibited* by

Table 3 Top 20 canonical pathways modified by A_{2A}AR KO in healthy myocardium

Canonical pathways	*P* value	Ratio	Genes
Relaxin signalling	3.55E-03	1.99E-02	*Gnb1, Prkar2b, Pde3b*
Cardiac β-adrenergic signalling	3.55E-03	2.11E-02	*Gnb1, Prkar2b, Pde3b*
Cellular effects of sildenafil (Viagra)	3.98E-03	1.99E-02	*Acta1, Prkar2b, Pde3b*
Protein Kinase A signalling	6.03E-03	1.26E-02	*Gnb1, Prkar2b, Pde3b, Nfatc2*
Phototransduction pathway	6.03E-03	3.08E-02	*Gnb1, Prkar2b*
Germ cell-sertoli cell junction signalling	6.31E-03	1.88E-02	*Iqgap1, Acta1, Map3k2*
Calcium signalling	8.71E-03	1.47E-02	*Prkar2b, Nfatc2, Acta1*
Glutamate receptor signalling	9.55E-03	2.86E-02	*Gnb1, Slc38a1*
Caveolar-mediated endocytosis signalling	1.41E-02	2.35E-02	*Acta1, Map3K2*
Nitric oxide signalling in the cardiovascular system	1.48E-02	2.02E-02	*Prkar2b, Pde3b*
Leptin signalling in obesity	1.55E-02	2.38E-02	*Prkar2b, Pde3b*
Cardiac hypertrophy signalling	1.78E-02	1.22E-02	*Gnb1, Prkar2b, Map3k2*
TR/RXR Activation	2.09E-02	2.02E-02	*Hp, Pde3b*
SAPK/JNK signalling	2.14E-02	1.98E-02	*Gnb1, Map3k2*
Colorectal cancer metastasis signalling	2.14E-02	1.17E-02	*Gnb1, Prkar2b, Ptger3*
α-Adrenergic signalling	2.19E-02	1.89E-02	*Gnb1, Prkar2b*
RANK signalling in osteoclasts	2.19E-02	2.04E-02	*Nfatc2, Map3K2*
G Beta Gamma signalling	2.24E-02	1.68E-02	*Gnb1, Prkar2b*
IL-1 signalling	2.29E-02	1.89E-02	*Gnb1, Prkar2b*
fMLP signalling in neutrophils	2.95E-02	1.57E-02	*Gnb1, Nfatc2*

Canonical signalling paths modified by A_{2A}R KO are shown, ranked according to *P* values determined by a Fisher's Exact Test. Also shown is a ratio value reflecting the number of molecules in a given path that meet cut-off criteria for differential expression, divided by the total number of molecules in the pathway

A_{2A}R KO, *Rac2* was implicated in 34, *Nfkbie* in 21, *Prkar2b* in 10, *Hmox1* and *Eif2ak2* each in 6 and *Eif4ebp1* and *Cyp2b6* each in 5 (Tables 6 and S10). These data point to an influence of intrinsic A_{2A}R activity on key signalling responses, including G protein, PKA, STAT1 and NFκB-dependent processes (see Supplementary material for additional discussion). While the absence of A_{2A}Rs impacted inflammatory and underlying signalling changes in endotoxemia, paths implicated in endotoxemic cardio-depression (ß-adrenergic and PKA signalling, Ca^{2+} handling, excitation-contraction coupling and mitochondrial function; Figs. S18–S21) were not among those sensitive to A_{2A}R KO (Table 6), consistent with a lack of effect of A_{2A}R KO on contractile dysfunction itself (Table 1).

PCR analysis of select transcripts

Quantitative RT-PCR analysis supports microarray-determined changes in transcripts selected for differential responsiveness to LPS and A_{2A}R KO (Fig. 7). While expression ratios vary slightly between the two methods, a significant linear relationship was apparent between both measures (with a slope > 1 suggesting greater dynamic range for RT-qPCR measurement).

Discussion

The present study characterizes impacts of A_{2A}R deletion on the myocardial transcriptome, markers of inflammation and cardiac function and injury in healthy and endotoxemic mice. Data indicate A_{2A}R activity exerts limited effects in un-stressed myocardium, with transcriptomic changes limited to G protein, cAMP/PKA and cGMP/NO signalling downstream of this and other GPCRs (Tables 2 and 3). However, during endotoxemia, absence of A_{2A}Rs exaggerated myocardial injury (and age- and sex-dependent mortality), without substantially modifying patterns of cytokine release, myocardial cytokine/chemokine transcription or contractile depression. The latter is consistent with insensitivity of the 'cardio-depressant' profile in endotoxemic hearts to A_{2A}R KO. Rather, data reveal A_{2A}R activity selectively influences transcription of regulators of NFκB and JAK-STAT signalling during endotoxemia, which may limit myocardial inflammation

and injury. Additional changes with $A_{2A}R$ KO suggest potential influences on insulin-resistance, hypertrophy/remodelling and vascular control/angiogenesis in endotoxemia.

$A_{2A}R$ activity and the transcriptome in un-stressed myocardium

Modest impacts of $A_{2A}R$ KO in un-stressed hearts (Table 2) are consistent with a largely retaliatory or stress-responsive role for myocardial $A_{2A}Rs$. Indeed, deletion failed to modify cardiac or vascular function, and circulating CRP, haptoglobin and cytokines in healthy animals. Functional annotation of transcripts supports $A_{2A}R$-dependent shifts in relaxin, adrenergic, Ca^{2+}, PKA, SAPK/JNK and hypertrophic pathways (Tables 3 and S2), involved in cellular growth/movement/death, immune and cell-to-cell signalling, and toxicological processes of fibrosis, cell damage and inflammation. This profile stems from a handful of changes spanning pathways (i.e. *Gnb1*, *Nfat2c*, *Acta1*, *Prkar2b*, *Map3k2* and *Pde3b*), supporting effects of $A_{2A}R$ activity on G protein and cAMP/PKA signalling downstream of the receptor [5, 8, 60]. Deletion of the $A_{2A}R$ has been shown to reduce cAMP and PKA activation in other cell types [61], consistent with impacts of KO here (Table 3). The altered MEKK2 path is also linked to G protein/Rac-dependent signalling distal to this and other GPCRs. In terms of vasoregulatory functions of the $A_{2A}R$, shifts in inter-related pathways involved in relaxin signalling, cellular effects of sildenafil and NO signalling (Table 3) support modulation of cGMP/NOS dependent control, while cardiac arteriopathy was identified as a pathologic process sensitive to $A_{2A}R$ KO (Table 4). These rather limited transcriptomic changes in healthy myocardium are consistent with observations in other tissues. For example, Yu et al. [62] found $A_{2A}R$ KO alters a very small sub-set of transcripts in healthy striatum (implicating $A_{2A}R$ sensitive EGR-2 control), with expression changes also modest (a majority ≤twofold). Others report no impact of $A_{2A}R$ KO on myocardial expression of RAC1, ERK1/2, p38-MAPK or JNK [14], though phospho-activation of the latter kinases was impaired, potentially reflecting shifts in cAMP/PKA and MEKK2 signalling.

Transcriptomic profile of endotoxemic myocardium

Myocardial injury and dysfunction are critical determinants of circulatory changes and mortality with uncontrolled inflammation; however, their mechanistic basis is poorly defined. Transcriptomic interrogation can reveal elements of these complex responses, though there are few analyses of myocardial [41, 42, 49] or cardiomyocyte [43, 49] responses to endotoxin/sepsis. Approximately 15 % of the 25,646 transcripts expressed in murine hearts were modified in endotoxemia (Table S4), encompassing a multiplicity of canonical paths and functions (Tables 5, S5–S7). Many are consistent with those highlighted by Wong et al. in more limited analysis of endotoxemic rats [41] and Rudiger et al. in a rat faecal peritonitis model [49]. The myocardial response involves profound upregulation of immune/inflammatory paths (Figs. 3–5 and S4–S7), the most highly modified including acute-phase response, PRR/TLR and interferon/IRF signalling (Table 5). Underlying NFκB, JAK-STAT and MAPK/PI3K paths are up-regulated (Tables 5, S5 and S7; Figs. S8–S12), with NFκB/JAK-STAT mechanistically linking the top five canonical pathway responses. Integrated signalling via NFκB and JAK-STAT paths thus appears central to myocardial endotoxemia, as in other tissues. In other tissues, these paths are also targeted by $A_{2A}Rs$ to suppress inflammatory/immune responses [44, 45, 58], with KO augmenting NFκB activation in macrophages, for example [6].

Amplified cardiac TLR and interferon signalling Since uncontrolled inflammation induces cellular injury/death, pro-inflammatory TLR/CD14 signalling is the subject to negative control. However, this path was transcriptionally amplified in endotoxemic myocardium (Fig. 5), including receptor molecules (*Cd14*, *Tlr1*, *Tlr2*, *Tlr3* and *Tlr4*) and LPS-binding protein (*Lbp*), transduction molecules (*Cr3/Mac1* and *Cr4*), MyD88, TIRAP and MyD88 targets (*Il6* and *Il1b*), MyD88-independent signal components (*Tbk1*, *Cxcl10*, *Ccl2* and *Ccl5*) and downstream JAK-STAT signalling (Fig. S9). Upregulated TLR and MyD88-dependent and independent signalling are consistent with changes in rat sepsis [49], though *Tlr4*, *Cd14* and *Tirap* induction here was not apparent in the rat model. Sweeney et al. [63] recently reported a novel MyD88-independent path linking TLR2/TLR4 signalling to *Ppargc1a*, encoding the mitochondrial biogenesis co-regulator PGC-1α. However, despite induction of elements of this path here (including *Tlr2*, *Tlr3*, *Tlr4* and *Irf7*), cardiac *Ppargc1a* was repressed by LPS, a response potentially exaggerating mitochondrial dysfunction and countered by $A_{2A}R$ activity.

Interferon signalling transducing inflammatory/TLR responses was also upregulated (Fig. 5), including a majority of gene targets and the path of IRF activation by PRRs (Figs. S5–S7). This entailed induction of membrane (*Tlr2*, *Tlr3* and *Tlr4*), cytosolic (*Ddx58*, *Ifih1*, *Eif2ak2* and *Oas1*) and extracellular (*C1q*, *C3*, *C3a* and *Ptx3*) receptors, and downstream mediators

Table 4 Top 20 biological and toxicological functions modified by A_{2A}AR KO in healthy myocardium

Biological pathway	*P* values	Genes
Cellular development	7.34E-04–4.5E-02	*Mrap, Ctsk, Sacs, Prkar2b, Ptger3, Nfatc2, Tslp, Map3k2*
Cellular growth and proliferation	7.34E-04–4.01E-02	*Mrap, Ppbp, Nfatc2, Tslp*
Haematological system development and function	7.34E-04–4.5E-02	*Hp, Ptger3, Ticam1, Ppbp, Nfatc2, Tslp, Map3k2*
Humoral immune response	7.34E-04–4.5E-02	*Nfatc2, Fmod, Tslp*
Cell-to-cell signalling and interaction	1.53E-03–4.74E-02	*Ank3, Ptger3, Ticam1, Ppbp, Nfatc2, Iqgap1, Tslp, Map3k2*
Amino acid metabolism	2.55E-03–5.09E-03	*Slc38a1*
Carbohydrate metabolism	2.55E-03–4.72E-02	*Gnb1, Pon1, Ppbp, Ppa1*
Cell death	2.55E-03–4.5E-02	*Ppbp, Nfatc2, Tslp, Map3k2*
Cell morphology	2.55E-03–3.68E-02	*Ank3, Ptger3, Nfatc2, Cnpy2, Iqgap1, Tslp*
Cellular assembly and organization	2.55E-03–4.01E-02	*Ank3, Ctsk, Nfatc2, Fmod, Cnpy2, Iqgap1, Acta1*
Cellular compromise	2.55E-03–4.01E-02	*Ank3, Hp, Ctsk, Ptger3, Ppbp*
Cellular function and maintenance	2.55E-03–1.52E-02	*Hp, Ppbp, Nfatc2, Tslp, Map3k2*
Cellular movement	2.55E-03–4.58E-02	*Gnb1, Ctsk, Hp, Ticam1, Ppbp, Nfatc2, Iqgap1, Tslp*
Connective tissue development and function	2.55E-03–3.02E-02	*Ctsk, Rpl22, Ptger3, Pde3b, Ppbp, Cidec, Nfatc2*
Connective tissue disorders	2.55E-03–5.09E-03	*Nfatc2*
Developmental disorder	2.55E-03–2.55E-03	*Ctsk*
Genetic disorder	2.55E-03–4.01E-02	*Ank3, Ctsk, Ptger3, Cidec, Iqgap1, Tslp, Mrap, Gnb1, Pon1, Hp, Prkar2b, Pde3b, Nfatc2, Dgat1, Acta1*
Hair and skin development and function	2.55E-03–5.09E-03	*Ptger3, Dgat1*
Immune cell trafficking	2.55E-03–3.51E-02	*Hp, Ticam1, Ppbp, Nfatc2, Tslp*
Inflammatory response	2.55E-03–4.67E-02	*Hp, Ptger3, Ticam1, Ppbp, Nfatc2, Fmod, Tslp*
Molecular toxicological function	*P* values	Genes
Liver fibrosis	3.01E-03	*Hp, Tslp*
Liver damage	1.43E-02	*Ticam1, Tslp*
Liver steatosis	2.45E-02	*Pde3b*
Cardiac arteriopathy	3.41E-02	*Ank3, Cux2, Dgat1, Gk5, Nsmce1, Pde3b, Pon1*
Cardiac infarction	3.76E-02	*Acta1, Pon1*
Liver hepatitis	4.41E-02	*Pde3b*
Hepatocellular carcinoma	4.43E-02	*Hp, Iqgap1*
Liver steatohepatitis	5.67E-02	*Pde3b*
Renal proliferation	7.90E-02	*Tslp*
Liver inflammation	9.36E-02	*Hp*
Pulmonary hypertension	1.08E-01	*Ptger3*
Nephrosis	1.10E-01	*Pde3b*
Cardiac congestive cardiac failure	1.63E-01	*Pde3b*
Heart failure	1.63E-01	*Pde3b*
Renal nephritis	2.50E-01	*Pde3b*

Enriched biological/toxicological functions of A_{2A}R KO sensitive transcripts are listed according to *P* values or ranges determined by a Fisher's Exact Test

(*Il6, Irf7* and *Rantes*). These shifts in TLR/CD14, MyD88-dependent and -independent and interferon/IRF signalling are relevant to temporal expansion of molecular changes [41, 42] and injury progression in

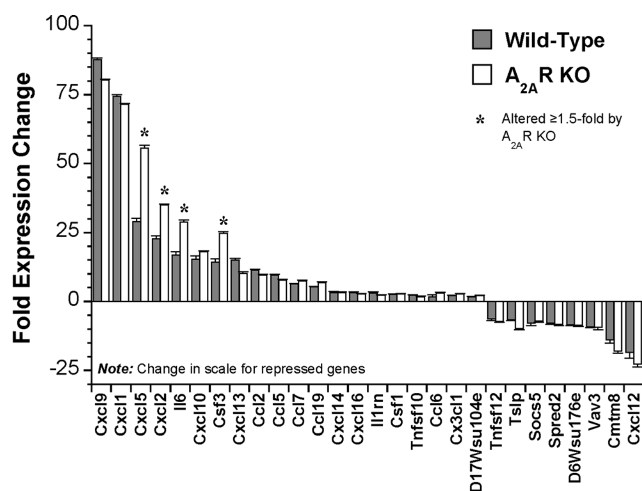


Fig. 3 The 25 most induced and 25 most repressed transcripts in hearts from young (2–3 month) mice challenged with LPS for 24 h. Mice were injected with 20 mg/kg LPS or saline vehicle and left ventricular tissue isolated for analysis 24 h later. Data from both wild-type and A_{2A}R KO hearts are shown for comparison (n = 6–8/group). Data are means ± S.E.M

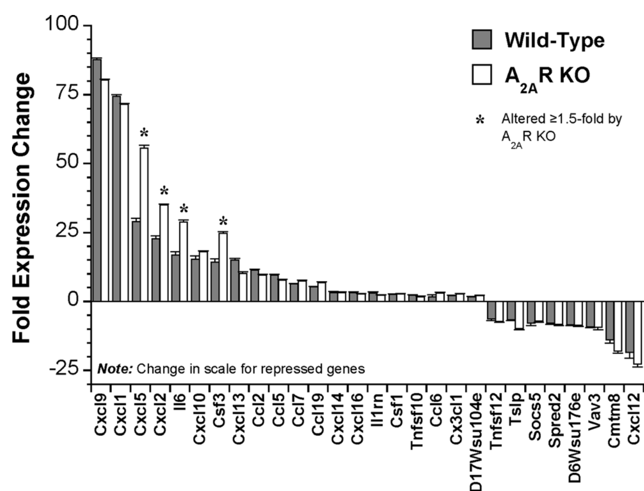


Fig. 4 Effects of A_{2A}R KO on the most LPS-responsive cytokines in wild-type hearts. Mice were injected with 20 mg/kg LPS or saline vehicle, and left ventricular tissue isolated for analysis 24 h later. Data from both wild-type and A_{2A}R KO hearts are shown for comparison (n = 6–8/group). Data are means ± S.E.M

endotoxemia: CD14 [64] and TLR4 [65, 66] both mediate cardiac dysfunction and injury and MyD88 signalling promotes cardiac hypertrophy [67], inflammation and injury [68, 69]. These profound responses diverge from in vitro data suggesting dampened/transient impacts of LPS on isolated myocyte NFκB and IκB kinase [43, 63].

Endotoxemic cardio-depression Cardiomyocyte and myocardial function is LPS-sensitive and depressed in endotoxemia [21, 23, 70, 71] (Table 1). This has been linked to abnormalities in myofibrillar Ca^{2+} sensitivity [70, 71], adrenergic control [71–74] and mitochondrial function [75], together with cell death [23, 76, 77]. Transient changes in preload may also mediate early reversible depression in vivo [78]. Transcriptomic data reveal a cardio-depressant profile entailing suppression of key determinants of contraction, including Ca^{2+}, β-adrenergic and PKA signalling, mitochondrial function and electromechanical coupling (Figs. S18–S21). Importantly, and despite other impacts, A_{2A}R expression did not influence this cardio-depressant profile nor modify contractile depression (Table 1) [23].

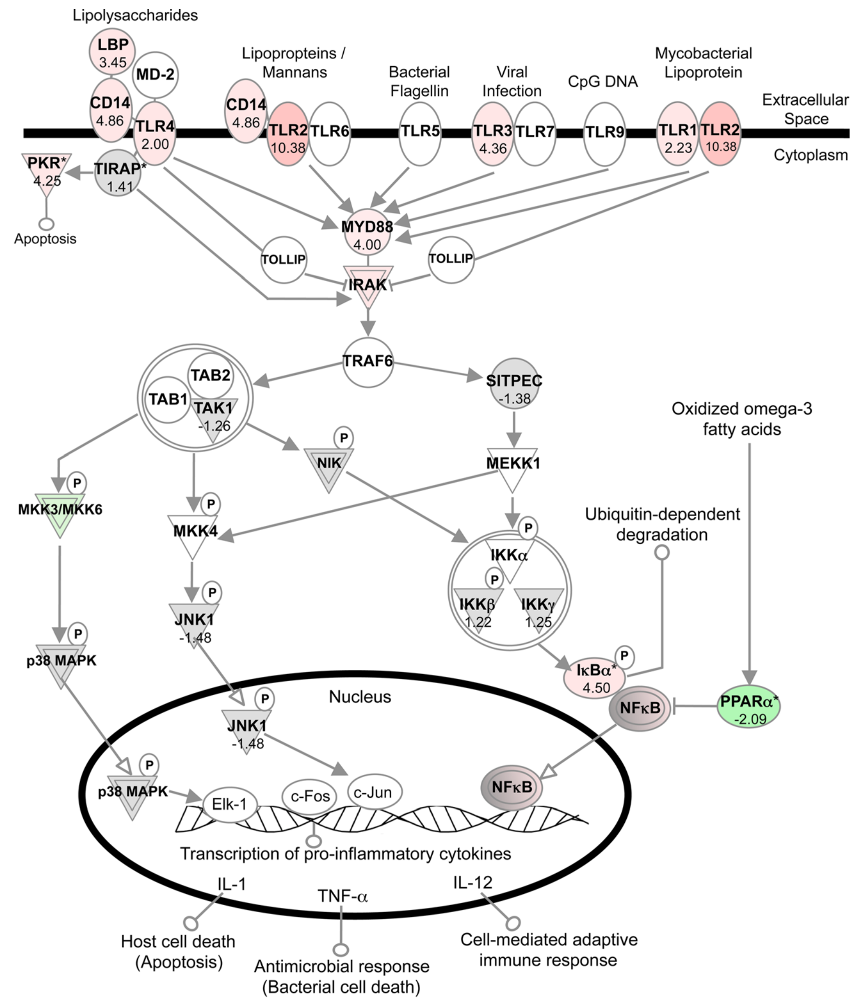
Suppression of β-adrenergic and Ca^{2+} signalling is consistent with desensitization in hearts and myocytes [71–73], and transcriptional changes in a rat model of faecal peritonitis [49]. Despite overlap with the latter response, the β_1- rather than β_2-adrenoceptor is depressed in endotoxemic mouse heart (Fig. S18), while reductions in AKAP, PP1, PP2A, NCX and L-type Ca^{2+} channel components observed here were not evident in the peritonitis model. Nonetheless, data collectively implicate β-adrenergic dysfunction as a common component of cardio-depression, consistent with reductions observed in β-adrenoceptor expression [73], tissue noradrenaline and adrenaline [73] and adrenoceptor stimulation of cAMP and Ca^{2+} fluxes [73]. In terms of approaches to inotropic support, coincident depression of adrenoceptor, PKA and Ca^{2+} signalling may limit the value of interventions targeting these effectors. Abnormalities within the contractile apparatus itself (repressed α-tropomyosin, troponin-T, α-actin and titin transcription) may also be relevant. Targeting mitochondrial dysfunction may more broadly improve cardiac outcomes: repression of Complex I components (Fig. S21) is consistent with Complex I specific dysfunction and ROS generation in mitochondria from endotoxemic hearts [75, 79].

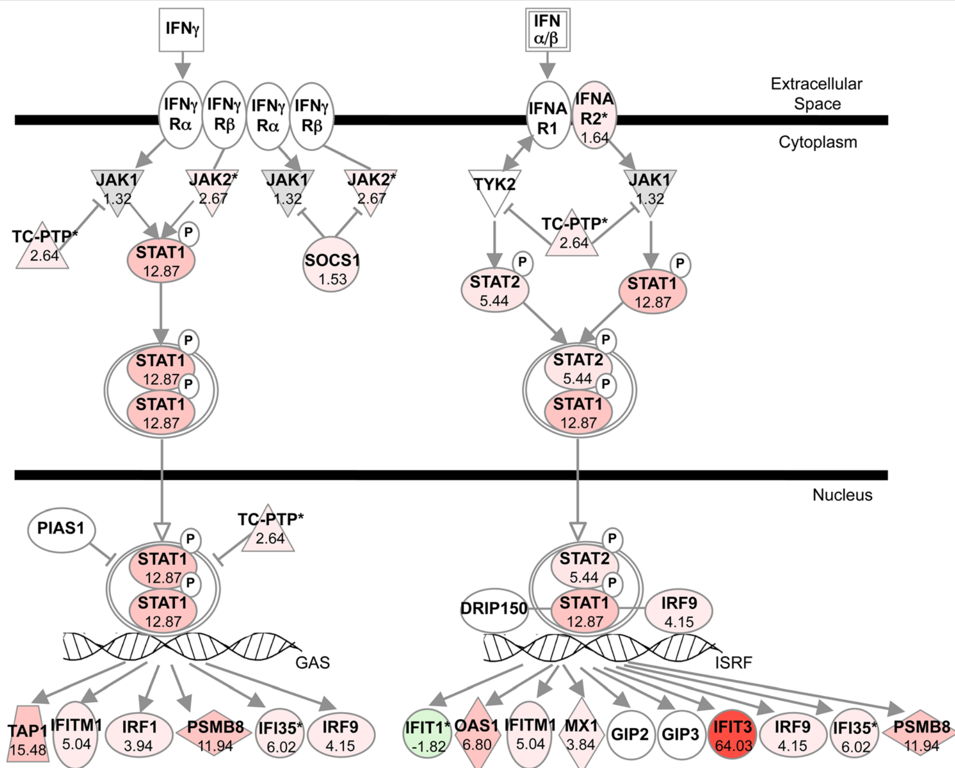
Additional cardio-depressant changes include altered TLR4 and fibroblast factor signalling, suppression of substrate metabolism and IGF-1 signalling (Fig. S22) and induction of inhibitory *S100a8/S100a9*, *Icam1*, *Vcam1*, *Cybb* (NOX2), *Ptgs2* (COX2) and the TNF-α receptors *Tnfr1* and *Fas*. Marked induction of S100 A8 and S100 A9 (TLR4 activators promoting endotoxemic injury) has been observed in LPS-treated myocytes, involving MyD88 and NFκB signalling [80]. Intriguingly, two adipokines with opposing actions, relevant to cardiac dysfunction, were among the most responsive to LPS. Pro-inflammatory and injurious lipocalin-2 (*Lcn2*) was the most induced (~600-fold), consistent with changes in rodent and human myocarditis [59], while anti-inflammatory and cardioprotective adiponectin (*Adipoq*) was the second most repressed. Lipocalin-2 is induced via IκBζ (+13 with LPS) and with heart failure, ischemia and inflammation [81, 82]. Profound induction

Table 5 Top 20 canonical pathways modified by LPS (≥2-fold change, <1 % FDR) in WT hearts

Canonical pathways	*P* value	Ratio	Genes
Acute phase response signalling	9.12E-09	2.02E-01	*Socs3, Rac2, Hamp, Serping1, Nfkbie, Socs2, Cp, Saa2, Serpina3, Jak2, Il6, Rbp1, Nr3C1, C1r, Hmox1, Shc1, Nfkbia, Cfb, Lbp, Serpine1, Nfkbib, Saa1, C3, Tnfrsf1a, Myd88, C1s, Rac1, Serpinf1, Cebpb, Stat3, Nfkb2, Hp, Rras2, Il1Rn, C4a, C2*
Interferon signalling	1.82E-08	4.33E-01	*Ifit3, Oas1, Ptpn2, Mx1, Ifi35, Irf9, Psmb8, Jak2, Tap1, Irf1, Ifitm1, Stat2, Stat1*
Activation of IRF by cytosolic pattern recognition receptors	1.38E-07	2.33E-01	*Dhx58, Nfkbie, Zbp1, Irf9, Tbk1, Il6, Nfkb2, Adar, Isg15, Ifih1, Irf7, Nfkbia, Ddx58, Stat2, Nfkbib, Stat1, Ifit2*
Hepatic fibrosis/hepatic stellate cell activation	7.94E-07	2.01E-01	*Icam1, Lepr, Myh7b (Includes Eg:668,940), Myh11, Ccl5, Il6, Fas, Pgf, Vegfa, Il1r2, Cxcl3, Igf1, Cyp2e1, Timp1, Lbp, Stat1, Timp2, Egfr, Il4r, Vcam1, Tnfrsf1a, Vegfc, Igfbp5, Nfkb2, Myl7, Csf1, Cd14*
Dendritic cell maturation	9.12E-07	1.51E-01	*B2m, Rac2, Icam1, Lepr, Nfkbie, Pik3r5, Cd83, Il6, Jak2, Fcgr2b, Fcgr1a, Nfkbia, Hla-b, Tlr3, Stat1, Nfkbib, Fcgr3a, Hla-c, Myd88, Tnfrsf1a, Relb, Rac1, Nfkb2, Tlr2, Il1rn, Fcer1g, Stat2, Irf8*
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	2.57E-06	1.34E-01	*Socs3, Rac2, Icam1, Mmp3, Nfkbie, Pik3r5, Jak2, Ccl5, Il6, Il17ra, Fcgr1a, Pgf, Myc, C1r, Il1r2, Vegfa, Nfkbia, Traf3Ip2, Wif1, Cfb, Plcb1, Tlr3, Nfkbib, Prkd1, Fcgr3a, Calm1, Adamts4, Sele, Vcam1, Myd88, Tnfrsf1a, C1s, Daam1, Rac1, Vegfc, Stat3, Cebpb, Irak3, Tlr2, Hp, Rras2, Il1Rn, Csf1, Cxcl12, Cebpd, Sost, Wnt5a, Irak2*
Retinoic acid mediated apoptosis signalling	5.37E-06	1.94E-01	*Parp10, Art1, Tnfsf10, Parp3, Parp12, Parp9, Tiparp, Irf1, Parp4, Bid, Parp11, Cflar, Parp14*
Antigen presentation pathway	6.61E-06	2.56E-01	*B2m, Psmb9, Hla-e, Hla-b, Psmb8, Hla-g, Tap1, Tap2, Tapbp, Hla-c*
Role of pattern recognition receptors in recognition of bacteria and viruses	8.32E-06	2.12E-01	*Ptx3, Oas1, C3, Oas2, Myd88, Pik3R5, Il6, Nfkb2, Ccl5, Tlr2, Ifih1, Clec7a, Irf7, Syk, Ddx58, Eif2ak2, Tlr3*
Death receptor signalling	1.29E-05	2.34E-01	*Hspb3, Tnfrsf1a, Nfkbie, Tnfsf10, Tbk1, Nfkb2, Fas, Daxx, Nfkbia, Bid, Cflar, Nfkbib, Birc3, Birc2, Hspb1*
IL-10 signalling	3.24E-05	2.14E-01	*Ccr1, Socs3, Il4r, Nfkbie, Il6, Stat3, Nfkb2, Fcgr2b, Il1r2, Hmox1, Nfkbia, Il1rn, Cd14, Lbp, Nfkbib*
Role of RIG1-like receptors in antiviral innate immunity	6.61E-05	1.96E-01	*Dhx58, Ifih1, Irf7, Nfkbia, Nfkbie, Ddx58, Tbk1, Nfkb2, Nfkbib, Trim25*
IL-6 signalling	1.00E-04	1.94E-01	*Hspb3, Tnfrsf1a, Nfkbie, Il6, Nfkb2, Stat3, Cebpb, Jak2, Il1r2, Shc1, Nfkbia, Rras2, Il1rn, Cd14, Lbp, Nfkbib, Tnfaip6, Hspb1*
IL-8 signalling	1.02E-04	1.45E-01	*Rac2, Icam1, Cxcl1, Pik3r5, Pgf, Eif4ebp1, Vegfa, Hmox1, Gng11, Gna13, Nfkbib, Prkd1, Egfr, Vcam1, Rhoc, Rac1, Vegfc, Irak3, Cstb, Myl7, Myl9 (Includes Eg:98,932), Bcl2l1, Itgb2, Rras2, Ccnd2, Itgam, Irak2*
Bladder cancer signalling	1.26E-04	1.89E-01	*Fgf16, Mmp3, Fgf9, Mmp14, Mmp15, Vegfc, Pgf, Myc, Vegfa, Rras2, Mmp8, Fgf12, Cdkn1a, Chd1 (Includes Eg:1105), Rps6Ka5, Fgf7, Egfr*
Pathogenesis of multiple sclerosis	1.45E-04	5.56E-01	*Cxcl10, Ccr1, Cxcl9, Ccl5, Cxcl11*
Role of PKR in interferon induction and antiviral response	1.58E-04	2.39E-01	*Nfkbia, Tnfrsf1a, Nfkbie, Bid, Nfkb2, Eif2ak2, Tlr3, Stat1, Nfkbib, Fcgr1a, Irf1*
JAK/Stat signalling	2.00E-04	2.19E-01	*Rac2, Socs3, Pias2, Socs2, Rac1, Pik3r5, Stat3, Jak2, Shc1, Rras2, Cdkn1a, Cish, Stat2, Stat1*
Complement system	2.09E-04	2.5E-01	*C1R, Cfd, Serping1, C3, C1S, Cfb, C4a, Masp1, C2*
Glucocorticoid receptor signalling	2.14E-04	1.25E-01	*Rac2, Icam1, Nfkbie, Pik3R5, Pbx1, Ccl5, Jak2, Il6, Fcgr1a, Nr3c1, Il1R2, Shc1, Cxcl3, Hspa4, Nfkbia, Ar, Ccl13, Cdkn1c, Serpine1, Stat1, Fkbp5, Nfkbib, Sra1, Vcam1, Ccnh, Sele, Rac1, Stat3, Cebpb, Bcl2l1, Rras2, Il1rn, Cdkn1a, Fkbp4, Nr3c2*

may promote dysfunction given its inflammatory [83], pro-death [82, 84] and mitochondrial actions [84]. Current data suggest the $A_{2A}R$ may beneficially limit LPS-dependent IκBζ induction to suppress injurious lipocalin-2. Highly repressed adiponectin exerts anti-inflammatory, anti-oxidant and cardioprotective effects [85, 86], thus downregulation is also likely to promote inflammation and sensitize myocardium to oxidative-stress and cell death. Curiously, *Adipoq* repression was reduced with $A_{2A}R$ KO, suggesting a positive influence of $A_{2A}R$ activity on this response. These and other changes relevant to pathogenesis of cardiac depression


Lipolysaccharides
LBP 3.45
MD-2
CD14 4.86
TLR4 2.00
Lipopropteins / Mannans
CD14 4.86
TLR2 10.38
TLR6
Bacterial Flagellin
TLR5
Viral Infection
TLR3 4.36
TLR7
CpG DNA
TLR9
Mycobacterial Lipoprotein
TLR1 2.23
TLR2 10.38
Extracellular Space
Cytoplasm
PKR* 4.25
TIRAP* 1.41
Apoptosis
MYD88 4.00
TOLLIP
TOLLIP
IRAK
TRAF6
TAB2
TAB1
TAK1 -1.26
SITPEC -1.38
Oxidized omega-3 fatty acids
NIK
MEKK1
MKK3/MKK6
MKK4
Ubiquitin-dependent degradation
IKKα
IKKβ 1.22
IKKγ 1.25
JNK1 -1.48
p38 MAPK
IκBα* 4.50
NFκB
PPARα* -2.09
Nucleus
JNK1 -1.48
p38 MAPK
Elk-1
c-Fos
c-Jun
NFκB
Transcription of pro-inflammatory cytokines
IL-1
TNF-α
IL-12
Host cell death (Apoptosis)
Antimicrobial response (Bacterial cell death)
Cell-mediated adaptive immune response


IFNγ
IFN α/β
IFNγ Rα
IFNγ Rβ
IFNγ Rα
IFNγ Rβ
IFNA R1
IFNA R2* 1.64
Extracellular Space
Cytoplasm
JAK1 1.32
JAK2* 2.67
JAK1 1.32
JAK2* 2.67
TYK2
JAK1 1.32
TC-PTP* 2.64
STAT1 12.87
SOCS1 1.53
TC-PTP* 2.64
STAT2 5.44
STAT1 12.87
STAT1 12.87
STAT1 12.87
STAT2 5.44
STAT1 12.87
Nucleus
PIAS1
TC-PTP* 2.64
STAT1 12.87
STAT1 12.87
STAT2 5.44
STAT1 12.87
DRIP150
IRF9 4.15
GAS
ISRF
TAP1 15.48
IFITM1 5.04
IRF1 3.94
PSMB8 11.94
IFI35* 6.02
IRF9 4.15
IFIT1* -1.82
OAS1 6.80
IFITM1 5.04
MX1 3.84
GIP2
GIP3
IFIT3 64.03
IRF9 4.15
IFI35* 6.02
PSMB8 11.94

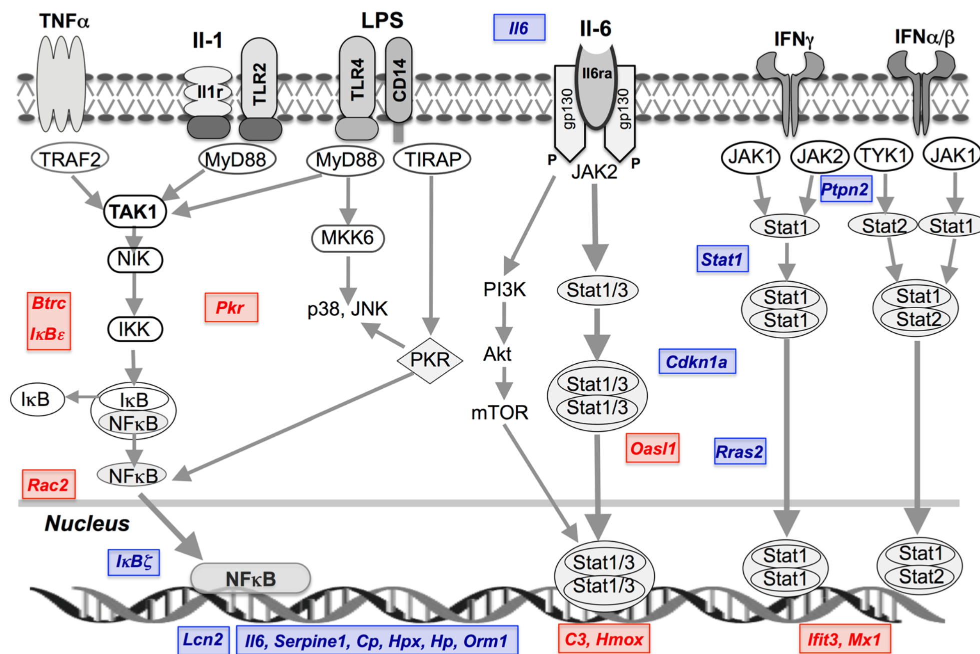


Fig. 6 Schematic of TLR, IL-1, IL-6 and interferon receptor activation of NFκB and JAK-STAT signalling and transcriptional control. The effects of A_{2A}R KO- on LPS-dependent gene changes are highlighted. These pathways are critical to cellular responses to LPS and orchestration of inflammation. Specific effects of A_{2A}R activity (based on effects of receptor KO) on transcriptional responses to LPS are highlighted (*red*—increased expression; *blue*—reduced expression)

are discussed further in the online Supplementary material.

A_{2A}R modulation of the endotoxemic transcriptome

Despite well-established anti-inflammatory effects of acute A_{2A}R agonism, the myocardial transcriptomic response to endotoxemia was largely insensitive to A_{2A}R KO (Figs. 3 and 4; Tables 7 and S9). Rather than a broadly suppressive impact of A_{2A}R activity, only 10 % of the transcriptomic response was modified by KO. This selective outcome is consistent with data for cytokine/chemokine transcription and release—the majority of these responses were also unaltered by A_{2A}R deletion, which specifically modified *Il6*, *Cxcl2*, *Cxcl5* and *Csf3* induction (Fig. 4) and circulating IL-5, haptoglobin and CRP levels (Figs. 1 and 2). These responses are nonetheless relevant to myocardial outcome: IL-6 initiates and expands inflammatory signalling, and CXCL2 and CXCL5 both participate in cardiac inflammation and remodelling. Important IL-6 target genes were highly induced with LPS, including *Bcl2l1*, *Cebpb*, *Irf1*, *JunB*, *Lbp*, *Socs3* and *Timp1*. By suppressing IL-6 transcription, potentially through IκBζ induction and STAT1 repression, A_{2A}R activity may limit cardiac IL-6 signalling. Functional annotation of A_{2A}R sensitive transcripts does support modulation of acute phase response, JAK-STAT, MAPK, PRR and NFκB pathways (Tables 7, S6 and S10). Figure 6 summarizes these key changes, highlighting impacts of A_{2A}R KO. Through modifying a small number of key transcript responses (*Nfkbie*, *Nfkbiz*, *Il6*, *Lcn2*, *Stat1*, *Cdkn1a* and *Rras2*), A_{2A}R activity may limit expansion of injurious inflammation in endotoxemic myocardium. In contrast, the cardio-depressant molecular profile appears insensitive to A_{2A}R KO.

NFκB signalling The A_{2A}R represses NFκB signalling via multiple mechanisms in non-cardiac cells, including phosphorylation-/SUMOylation-dependent IκB degradation and modulation of the SCF-E3 ubiquitin ligase complex [58]. The absence of A_{2A}Rs did modify the NFκB path in endotoxemic myocardium (Fig. 6), amplifying pro-inflammatory *Nfkbiz* (IκBζ) induction while countering inhibitory *Nfkbie* (IκBε) induction. Enhanced transcription of IκBζ and its targets *Lcn2* and *Il6* with A_{2A}R KO suggests the receptor may suppress inflammation by inhibiting IκBζ induction and its transcriptional actions, while promoting IκBε induction to limit nuclear translocation of NFκB (Fig. 6).

In contrast, A_{2A}R KO also reduced induction of molecules promoting NFκB signalling (Fig. 6): *Eif2ak2*/*Pkr* inhibits IκBα expression and enhances NFκB signaling and IFN-β expression; *Btrc* promotes degradation of IκBα and *Rac2* promotes cytokine-triggered NFκB signalling and IFN-γ expression. It is unclear what the balance of these changes might be, though repression

◀ **Fig. 5** Impact of 24 h LPS challenge on cardiac transcription of the TLR (upper panel) and interferon signalling paths (lower panel) in wild-type mice (n = 6–8/group). Induced transcripts highlighted in *red* and repressed in *green*. *Shaded transcripts* are altered by LPS by less than the threshold value

Table 6 Top canonical responses to LPS modified by A_{2A}AR KO (the 15 most promoted or inhibited by A_{2A}AR KO are shown)

Canonical Pathways	*P* value	Ratio	Genes
LPS responses enhanced by A_{2A}R KO (countered by A_{2A}R activity)			
Acute phase response signalling	7.41E-04	5.06E-02	*Hpx, Hp, Rras2, Pik3r1, Cp, Il6, Serpine1, Nr3c1, Orm1*
Glucocorticoid receptor signalling	9.33E-04	3.90E-02	*Hspa4, Cxcl3, Rras2, Pik3r1, Cdkn1a, Tgfb2, Il6, Stat1, Serpine1, Hspa2, Nr3c1*
Glioma invasiveness signalling	1.29E-03	8.47E-02	*Timp4, Rras2, Pik3r1, Rhoj, Itgb3*
Germ cell-sertoli cell junction signalling	1.45E-03	5.00E-02	*Rras2, Tuba8, Pak6, Map3k6, Pak3, Pik3r1, Tgfb2, Rhoj*
Angiopoietin signalling	3.16E-03	6.58E-02	*Grb14, Rras2, Pak6, Pak3, Pik3r1*
ILK signalling	3.55E-03	4.26E-02	*Ppp2r3a, Pik3r1, Vegfc, Atf4, Rhoj, Itgb6, Pgf, Itgb3*
Ephrin receptor signalling	3.98E-03	4.04E-02	*Rras2, Pak6, Pak3, Vegfc, Atf4, Epha4, Efnb3, Pgf*
ERK/MAPK signalling	4.37E-03	4.10E-02	*Rras2, Pak6, Pak3, Ppp2r3a, Ppp1R3c, Pik3r1, Atf4, Stat1*
Pancreatic adenocarcinoma signalling	4.47E-03	5.08E-02	*Pik3r1, Cdkn1a, Tgfb2, Vegfc, Stat1, Pgf*
HER-2 signalling in breast cancer	5.13E-03	6.17E-02	*Rras2, Pik3r1, Cdkn1a, Itgb6, Itgb3*
Macrophage NO & ROS production	7.08E-03	3.74E-02	*Map3k6, Ppp2r3a, Ppp1r3c, Pik3r1, Rhoj, Irf8, Stat1*
Hepatic fibrosis/stellate cell activation	1.05E-02	4.48E-02	*Cxcl3, Tgfb2, Vegfc, Il6, Stat1, Pgf*
Circadian rhythm signalling	1.12E-02	8.57E-02	*Per3, Arntl, Atf4*
HIF1α signalling	1.38E-02	4.55E-02	*Rras2, Pik3r1, Vegfc, Slc2a4, Pgf*
JAK/Stat signalling	1.41E-02	6.06E-02	*Rras2, Pik3r1, Cdkn1a, Stat1*
LPS responses inhibited by A_{2A}R KO (promoted by A_{2A}R activity)			
PXR/RXR activation	6.46E-04	4.4E-02	*Rac2, Prkar2b, Cyp2b6*
Nicotinate and nicotinamide metabolism	3.98E-03	2.94E-02	*Cilp, Art5, Eif2ak2, Bst1*
Amyloid processing	4.57E-03	5.08E-02	*Rac2, Prkar2b, Capn10*
Glutamate receptor signalling	5.89E-03	4.29E-02	*Glul, Slc38a1, Homer1*
AMPK signalling	9.55E-03	2.40E-02	*Rac2, Prkar2b, Adipoq, Eif4ebp1*
PRRs in recognition of bacteria & viruses	9.77E-03	3.66E-02	*C3, Ddx58, Eif2ak2*
NF-κB signalling	1.15E-02	2.58E-02	*Rac2, Nfkbie, Btrc, Eif2ak2*
NF-κB activation by viruses	1.23E-02	3.61E-02	*Rac2, Nfkbie, Eif2ak2*
Inhibition of angiogenesis by TSP1	1.55E-02	5.56E-02	*Rac2, Sdc1*
Role of RIG1-like receptors in antiviral innate immunity	1.66E-02	3.92E-02	*Nfkbie, Ddx58*
FXR/RXR activation	1.78E-02	2.91E-02	*Rac2, Sdc1*
TR/RXR activation	1.78E-02	3.03E-02	*Rac2, Ucp1*
PPARα/RXRα activation	1.86E-02	2.22E-02	*Prkar2b, Nfkbie, Adipoq*
RANK signalling in osteoclasts	1.95E-02	3.06E-02	*Rac2, Nfkbie, Map3k2*
Fcγ receptor-mediated phagocytosis in macrophages and monocytes	2.04E-02	2.97E-02	*Hmox1, Rac2, Hck*

Canonical pathways modified by LPS, ranked according to P-values determined by Fisher's Exact Test. Also shown is the ratio reflecting the number of molecules in a given path that meet cut-off criteria for differential expression, divided by the total number of molecules in the path

Table 7 Functional groupings of genes for which LPS responses were modified by ≥1.5-fold (induction or repression) by $A_{2A}R$ KO

Functional grouping	*P* value	Number of genes
LPS responses enhanced by $A_{2A}R$ KO (countered by $A_{2A}R$ activity)		
Molecular and cellular functions		
Cellular Movement	1.10E-08–5.10E-03	58
Cell-To-Cell Signalling and Interaction	1.78E-06–5.40E-03	46
Cell Death	1.13E-05–4.87E-03	74
Cellular Growth and Proliferation	1.30E-05–5.40E-03	58
Cell Cycle	2.22E-05–4.86E-03	30
Physiological system development and function		
Haematological System Development and Function	3.17E-07–5.40E-03	53
Haematopoiesis	3.17E-07–4.79E-03	36
Tissue Morphology	3.17E-07–4.79E-03	38
Immune Cell Trafficking	3.90E-07–5.10E-03	37
Cardiovascular System Development and Function	1.54E-06–4.25E-03	24
Disease and disorders		
Cancer	3.71E-08–5.03E-03	82
Immunological Disease	9.17E-08–3.81E-03	72
Skeletal and Muscular Disorders	7.90E-07–3.81E-03	77
Inflammatory Response	1.78E-06–5.05E-03	46
Haematological Disease	2.44E-06–3.68E-03	37
LPS responses inhibited by $A_{2A}R$ KO (promoted by $A_{2A}R$ activity)		
Molecular and cellular functions		
Carbohydrate Metabolism	2.24E-05–2.33E-02	6
Molecular Transport	2.38E-05–2.61E-02	24
Small Molecule Biochemistry	2.38E-05–2.87E-02	34
Lipid Metabolism	4.28E-05–2.87E-02	14
Cellular Compromise	6.16E-05–2.61E-02	11
Physiological system development and function		
Cardiovascular System Development and Function	4.28E-05–2.97E-02	5
Organ Development	4.28E-05–2.33E-02	7
Organismal Functions	9.49E-05–2.61E-02	4
Haematological System Development and Function	2.54E-04–3.11E-02	13
Lymphoid Tissue Structure and Development	2.54E-04–2.61E-02	6
Disease and disorders		
Inflammatory Response	6.16E-05–3.11E-02	15
Nutritional Disease	8.22E-05–6.58E-03	10
Genetic Disorder	1.34E-04–3.00E-02	30
Developmental Disorder	2.54E-04–2.61E-02	14
Neurological Disease	2.54E-04–2.61E-02	30

Functional groupings of LPS-responsive cardiac transcripts modified by $A_{2A}R$ KO (enhanced or repressed) by a factor of ≥1.5-fold. Functional groups derived from IPA analysis are categorized into molecular and cellular functions, physiological system development and function, and disease and disorders, and ranked according to *P* value ranges determined by a Fisher's Exact Test. Total numbers of involved genes are also shown

of the distal transcriptional effector IκBζ is predicted to limit effects of up-stream changes. Failure of $A_{2A}R$ KO to modify changes in genes recently implicated in cardioprotective effects of NFκB signalling [47] (including induction of *Ptx3*, *Plscr1*, *Sfi1* and *Igfbp3* and repression of *Car3*, *Dkk3, ai605517* and *Grhl2*) suggests receptor activity might selectively modify injurious rather than protective aspects of NFκB signalling.

JAK-STAT signalling In non-cardiac cells, $A_{2A}R$ agonism also inhibits JAK-STAT signalling, via control of SOCS transcription [58] and ubiquitination/degradation of JAK-

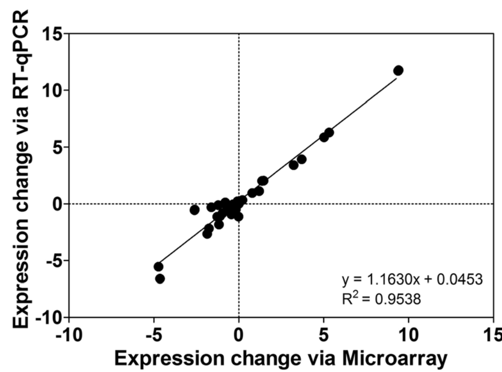


Fig. 7 Comparison of expression values for select transcripts assessed via array analysis and quantitative real-time PCR. Expression ratios are relative to values in wild-type (WT) hearts treated with normal saline (NS) and are thus shown for WTs treated with LPS for 24 h (WT LPS) and $A_{2A}R$ KO hearts untreated (KO NS) or treated with LPS (KO LPS). Data are means ± S.E.M, *n* = 6–8/group

phosphorylated STATs [87]. Despite no evidence for $A_{2A}R$ modulation of *Socs3* (induced with LPS unaltered by KO), $A_{2A}R$ deletion did modify the JAK-STAT response, exaggerating induction of both *Stat1* and the key activator *Il6*. Since STAT1 is crucial to LPS-induced apoptosis in non-cardiac cells [88], and mediates injury with ischemic insult [39], $A_{2A}R$-dependent suppression is predicted to be protective. Deletion of $A_{2A}Rs$ also enhanced endotoxemic induction of *Cdkn1a/p21Cip1/Waf1* (modulator of STAT1-dependent apoptosis and IFN-γ/STAT signalling, linked to poor sepsis outcomes [49]) and the signal transducer *Rras2*. These data collectively support beneficial $A_{2A}R$ modulation of JAK-STAT activation in endotoxemic myocardium (Fig. 6).

Select impacts on acute phase response vs. TLR and interferon signalling The acute phase response is triggered by IL-1, IL-6 and TNF-α and transduced via NFκB and JAK-STAT signals. Additional to effects of KO on *Il6* and JAK-STAT and NFκB paths, $A_{2A}R$ activity appears to counter induction of key gene targets (*Serpine1*, *Il6*, *Cp*, *Hpx*, *Hp* and *Orm1*). Conversely, $A_{2A}R$ activity was also associated with greater induction of target genes *C3* and *Hmox* and reduced induction of the glucocorticoid receptor inhibiting this inflammatory response. While mixed, collective shifts in JAK-STAT/NFκB paths support transcriptional suppression of the acute phase response via $A_{2A}R$ activity. However, while TLR/interferon paths were among the most highly responsive to LPS, $A_{2A}R$ KO did not substantially modify these responses (augmenting induction of MyD88-responsive *Irf8* and altering downstream NFκB signalling). Major changes in interferon signalling were also largely unmodified by $A_{2A}R$ KO (Fig. 6), suggesting select impacts of $A_{2A}R$ activity on elements of the acute phase response yet not associated TLR and interferon signalling.

Novel effects of $A_{2A}R$ KO The impact of $A_{2A}R$ activity is limited primarily to canonical elements of endotoxemia (Tables 6, S10 and S11); however, several of the more $A_{2A}R$-sensitive transcriptional responses to LPS hint at novel effects of $A_{2A}R$ signalling, including modulation of insulin-sensitivity, hypertrophic growth/remodelling and cardiac rhythmicity, together with influencing angiogenesis and vascular control.

Disruption of insulin-dependent glucose metabolism is an important consequence of endotoxemia, and three of the most $A_{2A}R$-sensitive transcripts (*Ptprf*, *Glut4/Slc2a4* and *Glut12/Slc2a12*) govern insulin-dependent glucose uptake (Table S9). Endotoxemic suppression of Glut4 and the secondary insulin-sensitive transporter Glut12 and the induction of *Ptprf* (encoding protein tyrosine phosphatase, receptor type F; implicated in insulin-resistance) were exaggerated >two-fold with $A_{2A}R$ KO (Table S9). Endotoxemic suppression of Glut4 and glucose uptake is reported in skeletal and cardiac muscle [89]. Conversely, increased expression of Glut4 improves glucose uptake and limits myocardial dysfunction in endotoxemia [89]. These responses to $A_{2A}R$ KO suggest regulatory influences of $A_{2A}R$ activity on glucose handling in endotoxemic tissue.

Other highly $A_{2A}R$-sensitive transcriptional responses suggest $A_{2A}R$ modulation of myocardial remodelling, including shifts in *Asb14* and *Asb15*, *Ca3* and *Ca4* and *Dnmt3a* and *Srpk2*. Endotoxemic repression of transcripts for 2 ankyrin repeat and SOCS box (ASB) proteins, *Asb14* and *Asb15*, was substantially exaggerated with $A_{2A}R$ KO. The ASB family bind and target proteins for degradation and regulate skeletal muscle development, while roles in heart are unclear. The ASB15 protein enhances skeletal muscle protein synthesis [90] and delays differentiation, potentially via modulating MAPK and PI3K/Akt signalling [91]. This control may be important in adaptive response to muscle load/activity. Carbonic anhydrases are also implicated in myocardial hypertrophy, with endotoxemic *Car4* induction (the dominant cardiac isoform) exaggerated and *Car3* downregulation was inhibited in $A_{2A}R$ KO hearts. The myocardial roles of these enzymes are only beginning to be unravelled, though they may promote hypertrophic responses by facilitating Na^+-H^+ and Cl-HCO_3 exchanger over-activities, while evidence from other cells suggests CAR3 may modify oxidative stress and apoptosis. Similarly, *Srpk2* induction was augmented in the absence of $A_{2A}Rs$, encoding a serine/arginine (SR) protein kinase (SRPK) that phosphorylates SR domain-containing proteins within interchromatin granule clusters/nuclear speckles to regulate pre-mRNA splicing. The SRPK2 protein also plays an

important role in cell proliferation and apoptosis. Additionally, downregulation of the methyltransferase transcript *Dnmt3a*, governing growth and function of embryonic myocytes [92], appears to be countered by $A_{2A}R$ activity, which may also limit cardiac fibrosis/remodelling [93]. Collectively, this suite of $A_{2A}R$ responsive changes may limit myocardial hypertrophy and remodelling responses to inflammatory insult. This is consistent with functional groupings sensitive to $A_{2A}R$ KO, including determinants of cell cycle, growth, proliferation and death and cardiovascular development (Table 7) and shifts in canonical hypertrophy pathways (Tables S10 and S11).

Curiously, $A_{2A}R$ activity also limited LPS induction of *Hcn1* (Table S9). The HCN proteins mediate the pacemaker (funny) current governing cardiac automaticity/excitability. While HCN4 is the most highly expressed, HCN1 is significantly expressed within the conduction network, contributes to cardiac rhythmicity [94] and is up-regulated in hypertrophy and heart failure. Endotoxemic induction may thus promote arrhythmicity, while $A_{2A}R$ activity appears to suppress this change.

Finally, $A_{2A}R$ KO modified a broader range of vascular control and growth pathways in endotoxemic (vs. healthy) hearts. Vascular dysfunction is a critical to organ damage and mortality and may involve NOS overactivity in some vascular beds. Deletion of the $A_{2A}R$ modified NO signalling together with renin-angiotensin signalling (Table S10), while cardiac arteriopathy was identified as a pathological process sensitive to KO (Table S11). These responses support influences of $A_{2A}R$ signalling on vascular pathology. We have previously shown endotoxemia impairs coronary hyperaemia, a dysfunction mimicked by KO of (and potentially involving) the $A_{2A}R$ [23]. Additionally, there is an apparent coronary 'over-supply' relative to myocardial demand in endotoxemic hearts (Table 1), owing to 25–30 % lower contractile function without reductions in coronary perfusion. The absence of coronary coupling to ventricular activity, described by us in this and other models [95, 96], suggests additional coronary dysregulation (though this effect is neither replicated nor modified by $A_{2A}R$ KO). Multiple paths/mediators regulating angiogenesis were also sensitive to $A_{2A}R$ KO in endotoxemic heart, including shifts in VEGF, HIF-1α, angiopoietin, TGF-β, GM-CSF, PDGF and IGF-1 pathways (Table S10) and key regulatory molecules (including exaggerated induction of *Il6*, *Pgf*, *Tgfb2*, *Tgfb2r*, *Angptl4* and *Amotl1*; exaggerated suppression of *Vegfc* and reduced suppression of *Qk*) (Table S9). While the A_{2B} receptor is more broadly implicated in control of angiogenesis, these changes suggest $A_{2A}R$-dependent processes may influence angiogenesis in the context of uncontrolled inflammation.

RT-qPCR confirmation of microarray-detected responses

Data confirm agreement between transcript expression determined via RT-qPCR and microarray methods (Fig. 7), with PCR further highlighting distinct transcriptional effects of $A_{2A}R$ KO: in some cases, KO eliminates responses to LPS (*Acta1*); alters baseline yet not LPS-dependent expression (*Amid* or *Dbp*); enhances induction (*Txnip*) or repression (*Cidec*) in response to vehicle and LPS or reduces gene expression in the vehicle group while negating further changes with LPS (*Eif4ebp2* and *Slc38a1*). Confirming the microarray approach, some of these responses are also functionally relevant. For example, *Txnip* encodes a stress-responsive protein inhibiting the anti-oxidant/signalling molecule thioredoxin. Reductions in TXNIP inhibit vascular inflammation and TNF-α signalling [97], TXNIP dysregulation promotes inflammatory disease [98] and elevations in TXNIP facilitate apoptosis [99]. Inhibition of *Txnip* induction by $A_{2A}R$ activity may thus enhance protective thioredoxin functionality.

Study limitations

Several study limitations are worth noting. While essential genesis of cardiac injury is studied and understood within the in situ organ, this entails experimental limitations inherent to in vivo studies of organ dysfunction in sepsis/endotoxemia [15, 16, 20, 21, 24, 41, 42, 49]. Organ injury in these settings not only involves intrinsic organ-specific mechanisms but also influenced by extrinsic factors, including shifts in systemic inflammation, haemodynamics and neuroendocrine influences (with relative impacts of these factors difficult to delineate). Although we assess the intrinsic myocardial dysfunction in ex vivo tissue (together with in vivo markers of cardiac damage), we cannot isolate potential influences of $A_{2A}R$ KO on cardiac loading, neurohumoral factors or indeed systemic inflammation. That said, while $A_{2A}R$ KO augmented some inflammatory markers, systemic and cardiac cytokine responses were largely insensitive to KO and the myocardial response was very selectively modified, indicating distinct effects of $A_{2A}R$ activity on cardiac injury processes. We are also unable to detail cell-specific origins of myocardial transcriptional changes. Migrating inflammatory cells could influence the transcriptomic profile for intact myocardium, though, as detailed in the Supplementary material, the expression profile of endotoxemic myocardial tissue is inconsistent with major contamination from such cells. An added limitation relates to the intriguing observation of both age- and sex-dependent mortality effects of LPS and $A_{2A}Rs$ (Fig. S3). This unfortunately precluded any detailed interrogation of these outcomes

due to poor survival in older KO mice. Future work might focus specifically on responses to LPS/sepsis in clinically relevant aged cohorts, correlating age-dependent transcriptomic and phenotypic outcomes. Finally, while profound transcriptional changes observed in the current profiling study are predicted to translate to functionally relevant protein changes, this exploratory analysis does not directly confirm altered protein expression.

Conclusions

Deletion of the $A_{2A}R$ induces modest effects in healthy myocardium, shifting transcription of G protein coupled cAMP/PKA, MEKK2 and cGMP/NO signalling downstream of this and other GPCRs, without impacting cardiac function. However, the receptor may play a retaliatory role in selectively suppressing inflammatory signalling and injury, yet, not contractile depression, in endotoxemic myocardium. Cardiac endotoxemia itself is characterized by profound upregulation of acute-phase response, PRR/TLR and interferon signalling and cell death and remodelling pathways, together with broad suppression of primary determinants of contractility (Ca^{2+}, ß-adrenergic and PKA signalling; mitochondrial function; electromechanical coupling) and induction of cardio-depressant genes (*Lcn2*, *S100a8/S100a9*, *Icam1*, *Vcam1*, *Nox2*, *Cox2*, *Tnfr1* and *Fas*). This complex transcriptome is selectively $A_{2A}R$ sensitive, with effects of KO implicating modulation of key regulatory molecules (*Nfkbie*, *Nfkbiz*, *Il6*, *Lcn2*, *Stat1*, *Cdkn1a* and *Rras2*) to suppress myocardial JAK-STAT/NFκB and pro-inflammatory IL-6 and TLR signalling, together with influencing determinants of insulin-sensitivity, hypertrophy/remodelling and vascular function/angiogenesis. In contrast, endotoxemic suppression of β-adrenergic, PKA, Ca^{2+} signalling, electromechanical and mitochondrial function pathways appears insensitive to $A_{2A}R$ KO, as does contractile dysfunction itself.

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Authors' contributions Conceived and designed the experiments: MER, KJA, JPH, RRM, SJM. Performed the experiments: MER, KJA, RRM, BT. Analysed data: KJA, MER, JPH, RRM, BT, LD. Generated and characterized KO mice: CL, PAH. Contributed reagents/materials/analytical tools: RRM, CL, PAH, KJA, JPH. Wrote the paper: KJA, MER, JPH, RRM, LMD, SJM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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