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MSK1 regulates the transcription of IL-1ra in response to TLR activation in macrophages.

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Abstract

The activity of the pro-inflammatory cytokine IL-1 is closely regulated *in vivo* via a variety of mechanisms, including both the control of IL-1 production and secretion as well as naturally occurring inhibitors of IL-1 function such as IL-1ra. IL-1ra is homologous to IL-1, and is able to bind but not activate the IL-1 receptor. IL-1ra can be produced by a variety of cell types, and its production is stimulated by inflammatory signals. Here we show that in macrophages the TLR mediated induction of IL-1ra from both its proximal and distal promoters involves the p38 and ERK1/2 MAPK cascades. In addition we show that mitogen and stress activated kinase 1 (MSK1) and 2, kinases activated by either ERK1/2 or p38 *in vivo*, are required for the induction of both IL-1ra mRNA and protein. MSKs regulate IL-1ra transcription via both IL-10 dependent and independent mechanisms in cells. Consistent with this, knockout of MSK in mice was found to result in a decrease in IL-1ra production following LPS injection. MSKs therefore act as important negative regulators of inflammation following TLR activation.

Keywords

MSK1, ERK1/2, NFκB, IL-1, IL-10, LPS

Introduction

Interleukin 1 (IL-1) is an inflammatory cytokine produced by the immune system that is critical in the host's response to infection. IL-1 activity is tightly regulated *in vivo*, both by control of IL-1 production and by levels of endogenous IL-1 inhibitory proteins. Mis-regulation of the IL-1 system has adverse consequences, and elevated IL-1 levels are implicated in several autoimmune diseases including rheumatoid arthritis and psoriasis (reviewed in [1-5]). The IL-1 system is made up of three proteins, IL-1 α , IL-1 β and IL-1ra, all of which bind with high affinity to IL-1 receptors (reviewed in [1, 6-9]). Both IL-1 α and IL-1 β are able to activate the type I IL-1 receptor resulting in the activation of several intracellular pathways including the NF κ B, p38 MAPK and JNK pathways [6, 7]. In contrast while IL-1ra can bind to the IL-1 receptor this binding is unable to activate downstream signalling. As IL-1ra competes with IL-1 for the IL-1 receptor *in vivo* it acts as a negative regulator of the IL-1 activity. In addition to IL-1ra, IL-1 receptor II (IL-1R2) is also able to inhibit IL-1 signalling. IL-1R2 acts a decoy receptor that can bind tightly to IL-1 but cannot initiate signalling as it lacks an intracellular TIR domain [1-2]. Maintaining the correct balance of IL-1 signalling is important for the correct function of the immune system, as an excess of IL-1 over IL-1ra can lead to autoimmune disease [1]. For instance, IL-1 α over-expressing transgenic mice develop symptoms similar to arthritis [10]. Consistent with this, knockout mice for IL-1 β are less susceptible to collagen induced arthritis, while knockout of the inhibitory IL-1ra in a BALB/cA genetic background can result in the spontaneous development of arthritis [11, 12]. The role of IL-1 in human autoimmune diseases has been confirmed by results from anti IL-1 therapies. Recombinant human IL-1ra (Anakinra) has been approved for the treatment of arthritis [3, 5, 13], and has shown promise in the treatment of systemic-onset juvenile idiopathic arthritis and adult onset Still's disease. A disadvantage of Anakinra is its poor pharmacokinetics, however improved biologics against IL-1, including monoclonal IL-1 β antibodies and IL-1 traps based on the IL-1 receptor are now being evaluated in the clinic.

The production of IL-1 and IL-1ra in the immune system is tightly controlled by both transcriptional and post-transcriptional mechanisms [14, 15]. Both IL-1 and IL-1ra are produced in response to a variety of signals including TNF, IL-1, phorbol esters and microbial products such as LPS [16, 17]. LPS, a component of gram-negative bacterial cell walls, stimulates the innate immune system via the Toll like receptor 4 (TLR4). Activation of TLR4 leads to the recruitment of the MyD88/Mal adaptor proteins and the activation of IRAKs. This results in the TRAF6 mediated activation of several intracellular pathways including the ERK1/2, p38 and JNK MAPK cascades as well as NF κ B pathway, all of which contribute to the transcriptional induction of cytokines. In addition TLR4 can also signal via a second adaptor, Trif, which is required for the activation of the TBK1/IRF3 pathway in response to LPS (reviewed in [18]).

In mice, the IL-1ra gene contains two alternative promoter regions, both of which are LPS responsive, which give rise to two distinct isoforms of IL-1ra [17, 19-22]. The distal promoter results in the production of a long form of IL-1ra that appears to be predominantly cytoplasmic, while the proximal promoter gives rise to a shorter secreted form. While luciferase reporter experiments have suggested that both distal and proximal promoters can be LPS regulated, only the proximal promoter has been extensively studied. Three major LPS responsive elements in the proximal promoter have been identified, a composite PU.1/NF κ B element, a potential C/EBP binding element and another PU.1 element [23-25]. In addition, an IL-10 responsive

STAT3 binding site and two IL-4 responsive STAT6 sites have been identified [26, 27]. Through the use of cell permeable inhibitors, a role for p38 in the LPS induced regulation of IL-1ra has been established [25], while ERK1/2 has been shown to be involved in IL-1ra transcriptional induction in response to leptin [28].

While both ERK1/2 and p38 are able to directly affect transcription via the phosphorylation of specific transcription factors, they are also able to induce transcription via activation of downstream kinases, which can be divided into 4 main groups: RSK, MSK, MNK and MAPKAP-K2/3 (reviewed in [29]).

Two isoforms of MSK, MSK1 and MSK2, have been identified in mammalian cells [30]. In cells MSKs are activated via a complex series of phosphorylation and auto-phosphorylation reactions downstream of ERK1/2 or p38 α [31, 32]. The main function identified for MSKs to date is the regulation of CRE dependent immediate early (IE) genes downstream of MAPK signalling [33]. Gene targeted mice that lack both MSK1 and MSK2 have demonstrated that these kinases are required for the mitogen and stress induced phosphorylation of the transcription factors CREB and ATF1 [34]. As a result, MSKs are involved in the regulation of several CRE dependent IE genes including MUC5AC, c-fos, NR4A1-3 and junB [35-38]. In addition to CREB, MSK can also phosphorylate the p65/RelA subunit of NF κ B, histone H3, and the chromatin associated protein HMG-14 [37, 39-42]. Surprisingly, MSK1/2 double knockout mice do not exhibit observable phenotype under normal conditions [34], although changes have been reported in specific behavioural algorithms [43-45]. Recently it has been found that MSK1/2 double knockout mice are more susceptible to LPS induced endotoxic shock, due to increased production of TNF, IL-6 and IL-12 but decreased production of IL-10 [46]. In response to LPS, production of IL-1 was not significantly altered by MSK knockout in either mice or isolated macrophages in these studies. The effect of MSK knockout on IL-1ra production was not however addressed in these studies [46]. Here we report that MSKs are involved in the transcriptional up-regulation of IL-1ra following TLR activation.

Methods

Antibodies

Antibodies against ERK1/2 were obtained from Cell Signalling Technology and the antibody against IL-1ra was from Santa Cruz Biotechnology.

Mice

Gene targeted mice that lack both MSK1 and MSK2 have been reported previously [33, 34]. IL-10 mice were obtained from the Jackson Laboratories [47]. All mice were backcrossed onto C57Bl6 for at least 12 generations before use. Animals were maintained under specific pathogen free conditions and in line with UK and EU regulations. Experiments approved by local ethical review and carried out under authority of a Home Office project licence.

Cell culture

Bone marrow derived macrophages were isolated as described [48]. Cells were maintained on bacterial grade plates for 1 week in DMEM supplemented with 10% heat inactivated FBS (Sigma), 2 mM L-glutamine, 100 units/ml penicillin G and 100 μ g/ml streptomycin 0.25 μ g/ml amphotericin (Invitrogen) and 5 ng/ml rCSF (R&D systems). Adherent cells were then re-plated on tissue culture plastic in fresh media and used 24 hours after re-plating.

Where indicated, cells were incubated with 100 ng of IL-10, 5 μ M SB203580, 2 μ M PD18435, 10 μ M BMS 345541 or 15 μ M PS 1145 or 10 μ g/ml cyclohexamide for 1

hour before use. Cells were stimulated with the TLR agonists LPS (Sigma, L6529), Type B CpG DNA (ODN1826, Invivogen) or Pam3CSK (Invivogen) for the indicated times.

The IL-10 neutralising antibody was used at 2.5 µg/ml and was a gift from Anne O'Garra (NIMR, London, UK). IgG isotype control (2.5 µg/ml) was from R&D Systems.

Quantitative RT PCR

RNA was isolated using the RNeasy Microkit from Qiagen. Total RNA was reverse transcribed using iScript (BioRad), and real time PCR carried out using Sybrgreen based detection methods. Primers for the mRNA studies are summarised in table I. For analysis of IL-1ra the primers set against exons 2/3 of the IL-1ra gene was used unless otherwise stated. For each PCR primer set the band obtained from amplification of reverse transcribed total mRNA was cloned and its sequence confirmed by DNA sequencing. The efficiency for each PCR primer set was also determined experimentally. For expression analysis 18s was used as a loading control and fold induction was determined from Ct values using the equation:

$$\text{relative mRNA level} = \frac{E_u^{(ct_{uc} - ct_{us})}}{E_r^{(ct_{rc} - ct_{rs})}}$$

where E is the efficiency of the PCR, ct is the threshold cycle, u is the mRNA of interest, r is the reference gene (18s RNA), s is the sample and c is the un-stimulated control sample.

Quantification of cytokine levels

Levels of IL-1ra secreted into the media were determined by an ELISA (R&D Systems) carried out according to the manufactures protocol. Concentrations were determined relative to a standard curve performed in the same assay. As this ELISA is directed against the C-terminal region of the protein it is likely to detect both secIL-1ra and icIL-1ra if present.

In vivo LPS injection

Mice were intraperitoneally injected with saline or LPS at 2.5 mg/kg (Sigma, L6529) and serum samples were collected 1h or 4 h after injection. Cytokine levels were determined by ELISA (R&D Systems).

Results

MSKs are required for the maximal induction of IL-1RA

Previous studies have shown that MSK1/2 double knockout does not affect the serum levels of IL-α or β following i.p. injection of LPS in mice [46]. It is however possible that MSKs could affect IL-1 signalling via the induction of IL-1ra. To test this, mice were given an i.p. injection of LPS and serum levels of IL-1ra measured by ELISA. This demonstrated that the induction of IL-1ra by LPS was significantly lower in MSK1/2 knockout mice compared to wild type animals (Fig 1A). LPS is also known to stimulate IL-1ra production in isolated macrophages via TLR4 [24], so the secretion of IL-1ra in primary bone marrow derived macrophages (BMDMs) from MSK1/2 knockout was examined. Similar to what was observed *in vivo*, MSK1/2 knockout BMDMs secreted less IL-1ra than wild type cells in response to LPS (Fig 1B).

As MSK's known functions relate to transcriptional regulation, the above results suggest a role for MSKs in regulating the IL-1ra promoter. Using Q-PCR it was found that LPS induced IL-1ra transcription in macrophages (Fig 2). Consistent with a role for MSKs, the maximal induction of IL-1ra was found to require p38 and

ERK1/2, the upstream activators of MSKs. Both the MEK1/2 inhibitor PD184352, which blocks the activation of ERK1/2, and the p38 α / β inhibitor SB203580 were found to partially inhibit the induction of the mRNA for IL-1ra, while addition of both inhibitors had a synergistic effect on the inhibition of IL-1ra mRNA induction (Fig 2A). This pattern of inhibition by PD184352 and SB203580 is similar to what has been reported for MSK1 activation by LPS [46]. To demonstrate a role for MSKs in IL-1ra transcription, BMDMs from MSK1/2 knockout mice were used. In response to LPS stimulation a significant reduction in IL-1ra transcription was observed in MSK1/2 knockout cells compared to wild type BMDMs (Fig 2B). This effect was not restricted to TLR4 stimulation, as similar results were obtained for the TLR2 agonist Pam3CSK (Fig 2C) and TLR9 agonist CpG DNA (Fig 2D).

MSK knockout could completely suppress IL-1ra transcription, suggesting that there was an additional, MSK independent mechanism contributing to the induction of this gene. One possibility for this is NF κ B. LPS also activates NF κ B, and NF κ B has previously been suggested to play a role in the transcription of IL-1ra [23, 49]. BMS 345541 or PS 1145, inhibitors of IKK that block NF κ B translocation to the nucleus, were able to inhibit IL-1ra transcription in wild type cells. In addition they were also able to block the residual induction of IL-1ra in MSK1/2 knockout cells (Fig 2E).

The IL-1ra gene contains two alternate first exons and promoter regions [20]. Transcription from the proximal promoter results in production of the secreted form of IL-1ra (secIL-1ra). Transcription from the distal promoter results in an intracellular form of the IL-1ra protein (icIL-1ra), and results in the use of an additional 5' exon that splices into an internal splice acceptor site in the 1st exon used by the proximal promoter (Fig 3A). The Q-PCR primers used in Fig 2 amplify a region encoded by exons 2 and 3, which is common to both IL-1ra isoforms. Two further PCRs were therefore designed to specifically amplify IL-1ra mRNA for either the proximal or distal isoforms (Fig 3A). Using these PCR reactions, LPS was found to stimulate transcription from both the proximal and distal promoters. In line with other reports, the relative Ct values (15.1 for secIL-1ra and 20.5 icIL-1ra following LPS stimulation) suggest that secIL-1ra (corresponding to transcription from the proximal promoter) was the major isoform transcribed in response to LPS. Analysis of MSK1/2 knockout cells showed that MSKs were required for the LPS induced transcription from both distal and proximal IL-1ra promoters (Fig 3B). Consistent with the results obtained for total IL-1ra mRNA induction (Fig 2B), the induction of IL-1ra transcription from the distal promoter and proximal promoters was blocked by a combination of PD184352 and SB203580 (data not shown), or by the IKK inhibitors BMS 345541 or 15 μ M PS 1145 (Fig 3C). Consistent with the effect of MSK1/2 knockout on IL-1ra mRNA induction, MSK knockout also affected IL-1ra protein levels. The secretory and intracellular forms of IL-1ra can be distinguished by their molecular weights. In wild type cells, induction of both isoforms could be seen in response to LPS. In both cases this was reduced by knockout of MSK1/2 (Fig 3D).

MSKs regulate IL-1ra via IL-10 dependent and independent mechanisms.

IL-1ra does not act as a classical immediate early gene in macrophages, as unlike classical IE genes such as nur77 and c-fos which show a rapid and transient induction, the induction of IL-1ra increases over time. In addition the induction of IL-1ra, but not that of TNF, after 6h of LPS treatment was inhibited by the protein synthesis inhibitor cyclohexamide, indicating that sustained induction of IL-1ra required *de novo* protein synthesis (Fig 4). One possible explanation for this is that in macrophages the LPS stimulated production of IL-10 acts as a positive feedback loop

to increase IL-1ra transcription. IL-10 and LPS have been described to have a synergistic effect on IL-1ra transcription, which has been suggested to occur via the IL-10 induced phosphorylation of STAT3, allowing the binding of STAT3 to a SBRE in the proximal IL-1ra promoter [26]. To examine this further, BMDMs were stimulated with LPS in the presence or absence of a neutralising antibody for IL-10. The use of an isotype control antibody had no effect on IL-1ra mRNA induction. The IL-10 neutralising antibody however reduced the induction of total IL-1ra in wild type cells (Fig 5A). In agreement with this we found that knockout of IL-10 reduced the induction of secIL-1ra mRNA in response to LPS (Fig 5B). Interestingly, IL-10 knockout also decreased the induction of icIL-1ra relative to wild type cells (Fig 5C). MSKs have previously been shown to be involved in the production of IL-10 following LPS stimulation [46, 50]. Decreased IL-10 production by the MSK1/2 knockout macrophages could therefore explain the decrease in IL-1ra mRNA levels relative to wild type cells. If this was the only mechanism by which MSKs could regulate IL-1ra transcription, addition of exogenous IL-10 to the cultures should normalise IL-1ra expression between the MSK1/2 knockout and wild type cells. BMDMs were therefore stimulated with either LPS, IL-10 or a combination of both IL-10 and LPS. The concentration of exogenous IL-10 used was in a 100 fold excess over the concentrations of endogenous IL-10 typically secreted by LPS stimulated BMDMs under the conditions used [46]. IL-10 alone was not a strong inducer of secIL-1ra transcription, however as expected, addition of exogenous IL-10 strongly potentiated IL-1ra induction by LPS in wild type cells (Fig 5D). IL-10 also synergistically increased LPS induced secIL-1ra mRNA levels in MSK1/2 knockout cells, however it was not able to restore secIL-1ra induction to the levels seen in wild type cells (Fig 5D). Similar results were obtained for icIL-1ra (Fig 5E). SOCS3 is another gene that is more strongly induced by a combination of LPS and IL-10 than LPS alone. In the same experiment IL-10 had a similar additive effect with LPS on SOCS3 induction in both wild type and MSK1/2 knockout cells (Fig 5F). To confirm that MSKs could regulate IL-1ra independently of IL-10 production, MSK knockouts were bred onto an IL-10 deficient background. Triple knockout of IL-10 and MSK1 and 2 resulted in a greater decrease in IL-1ra mRNA induction than IL-10 knockout alone (Fig 6). Together these results show that while MSKs may promote IL-1ra induction via an IL-10 autocrine feedback mechanism, MSKs are also capable of regulating IL-1ra transcription by an additional mechanism independent of IL-10.

Discussion

MSKs have previously been shown to play an important role in the negative regulation of TLR4 signalling through the induction of IL-10 and the MAPK phosphatase DUSP1 [46, 50]. Here we show that MSKs are also involved in the transcription of the anti-inflammatory cytokine IL-1ra.

The transcriptional regulation of IL-1ra is complex, and at least two alternate promoters exist for IL-1ra in mice, both of which are stimulated in macrophages by LPS. There is little sequence conservation between these regions, although both C/EBP and NF κ B sites have been described in both promoters [23-25, 49]. In response to LPS, the activity of both promoters is regulated by similar signal transduction cascades, including the ERK1/2, p38 and NF κ B pathways (Figs 2 and 3). In addition the activity of both promoters is stimulated more strongly by a combination of LPS and IL-10 than LPS alone (Fig 5). An effect of IL-10 on the IL-

1ra proximal promoter has previously been demonstrated [26], however the role of IL-10 in the regulation of the distal promoter has not previously been addressed. IL-10 activates JAKs resulting the phosphorylation of STAT3, and a functional STAT3 binding site has been identified in the proximal IL-1ra promoter [26]. STAT3 can bind to motifs corresponding to a TTNNNNAA consensus [51] and consistent with a role for STAT3 in the IL-1ra distal promoter, two mouse/human conserved potential STAT3 binding sites exist 1.5 kb upstream of the transcription start site in the distal promoter.

MSKs have previously been shown to regulate IL-10 production in response to LPS [46]. It is likely that the decrease in IL-10 secretion could contribute to some extent to the decrease in IL-1ra transcription in MSK1/2 knockout relative to wild type macrophages, especially at later time points. However it does not appear to be the major mechanism by which MSK regulates IL-1ra transcription for several reasons. MSK knockout affects IL-1ra transcription even at early time points, before significant release of IL-10 from the macrophages. Additionally, co-stimulation with LPS and exogenous IL-10 does not restore IL-1ra transcription in MSK knockouts. Finally knockout of MSK on an IL-10 deficient background still results in a decrease in LPS induced IL-1ra transcription.

The detailed mechanism by which MSK regulates IL-1ra will require further study. Most MSK dependent genes identified to date contain CRE elements (TGACGT(C/A)A), binding sites for the MSK substrates CREB and ATF1. No mouse/human conserved consensus CRE sites exist in the proximal or distal IL-1ra promoters, however it is possible that a CRE site could exist in an enhancer sequence separate from the promoters. Macrophages from a mouse with a knockin mutation of the MSK phosphorylation site in CREB have normal expression of IL-1ra (data not shown), however it is possible that compensation occurs in these mice from the closely related transcription factor ATF1, which is also phosphorylated by MSKs. Alternatively MSK may regulate IL-1ra transcription via a different substrate. Roles for both C/EBP and NF κ B have been described in IL-1ra transcription [23-25, 49]. C/EBP has been identified as a substrate for RSK [52, 53]. As RSKs and MSK are closely related and have overlapping substrate specificity [54], it is possible that MSKs may also phosphorylate C/EBP. MSKs have been shown to phosphorylate the p65/RelA subunit of NF κ B on Ser276 [39]. MSKs are not critical for NF κ B mediated transcription in response to LPS, as the transcription of many NF κ B dependent pro-inflammatory genes in MSK knockout macrophages is normal or enhanced in response to LPS [46]. Ser276 phosphorylation of NF κ B has however been described to have a promoter specific effect on NF κ B transactivation [55-57], and it is possible that IL-1ra is one of the genes regulated by Ser276 phosphorylation. It is also possible that MSK regulated IL-1ra via an unidentified mechanism.

In summary we report here that MSKs regulate the production of IL-1ra downstream of TLR signalling. Together with previous results showing that MSKs regulate IL-10 production in macrophages, these results identify MSKs as important mediators of anti-inflammatory cytokine production in the innate immune system.

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Figure legends

Fig 1 MSK regulates LPS induced IL-1ra secretion.

A) Wild type (black bars) or MSK1/2 knockout (white bars) mice were given an i.p. injection of either 2.5 mg/kg of LPS or saline. Plasma levels of IL-1ra were measured at 1 and 4 hours after LPS injection by ELISA. Error bars represent the standard deviation of measurements from 9 mice per genotype. B) BMDMs were isolated from both wild type (black bars) and MSK1/2 double knockout (white bars) mice as described in the methods. Cells were stimulated with 100ng/ml of LPS for the indicated times and the levels of IL-1ra secreted into the media measured by ELISA. Error bars represent the standard deviation from 3 independent cultures per genotype. Students t-test p-values of less than 0.01 between the wild type and knockout samples are indicated by **.

Fig 2 IL-ra transcription is regulated by MSKs and NFκB.

- A) BMDMs from wild type mice were incubated with 2 μM PD 184352 or 5 μM SB 203580 as indicated for 1h. Cells were then stimulated for 1h with 100 ng/ml of LPS. Total RNA was then isolated and IL-1ra mRNA levels determined by Q-PCR as described in the methods. Error bars represent the standard deviation from 3 independent cultures.
- B) BMDMs were isolated from wild type (black bars) and MSK1/2 knockout (white bars) mice. Cells were stimulated for the indicated times with 100 ng/ml LPS and the induction of IL-1ra determined by Q-PCR. Error bars represent the standard deviation of 3 independent cultures. Students t-test p-values of less than 0.05 between the wild type and knockout samples are indicated by * and less than 0.01 by **.
- C) As (B) except cells were stimulated with 100 ng/ml Pam-3-CSK for the indicated times and error bars represent the standard deviation of 4 independent cultures.
- D) As (B) except cells were stimulated with 2.5 μM CpG DNA for the indicated times and error bars represent the standard deviation of 4 independent cultures.
- E) BMDMs from wild type (black bars) or MSK1/2 knockout (white bars) mice were incubated with either 10 μM BMS 345541 or 15 μM PS 1145 as indicated for 1h. Cells were then stimulated for 1h with 100 ng/ml of LPS. Total RNA was then isolated and IL-1ra mRNA levels determined by Q-PCR as described in the methods. Error bars represent the standard deviation from 3 independent cultures.

Fig 3 MSK regulates both the distal and proximal IL-1ra promoters.

- A) The genomic structure of the IL-1ra locus is shown. Transcription from the proximal promoter gives rise to the secreted isoform of IL-1ra (secIL-1ra), which is encoded in 4 exons. Transcription from the distal promoter results the intracellular isoform (icIL-1ra) and makes use of an extra 5' exon. Splicing from this upstream exon (hatched box) occurs onto a splice acceptor internal to the 1st exon used by the proximal promoter. For this exon, the sequence in common is indicated in black, while the 5' sequence unique to the secreted isoform is shown in grey. Positions of the primers used for Q-PCR are indicated: P1 and P2 amplify both secreted and intracellular isoforms, P3 and P4 amplify the secreted isoform only and P5 and P6 the intracellular isoform only.

- B) BMDMs were isolated from wild type (black bars) and MSK1/2 knockout (white bars) mice. Cells were stimulated for the indicated times with 100 ng/ml LPS and the induction of secretory (secIL-1ra, upper panel) or cytoplasmic (icIL-1ra, lower panel) IL-1ra determined by Q-PCR. Error bars represent the standard deviation of 3 independent cultures. Students t-test p-values of less than 0.05 between the wild type and knockout samples are indicated by * and less than 0.01 by **.
- C) BMDMs from wild type mice were incubated with either 10 μ M BMS 345541 or 15 μ M PS 1145 as indicated for 1h. Cells were then stimulated for 1h with 100 ng/ml of LPS. Total RNA was then isolated and secretory (secIL-1ra, upper panel) or cytoplasmic (icIL-1ra, lower panel) IL-1ra mRNA levels determined by Q-PCR as described in the methods. Error bars represent the standard deviation from 3 independent cultures.
- D) BMDMs from wild type (black bars) or MSK1/2 knockout (white bars) mice and stimulated for the indicated times with 100 ng/ml LPS. Cells were lysed and the levels of IL-1ra protein determined by immunoblotting. Bands were detected using the fluorescence based Odyssey system (Li-Cor Biosciences). Representative blots are shown on the left, with quantification, using ERK1/2 as a loading control, on the right. Error bars represent the standard deviation of 3 independent cultures. Students t-test p-values of less than 0.01 between the wild type and knockout samples are indicated by **.

Fig 4 Sustained induction of IL-1ra requires protein synthesis.

BMDMs from wild type mice were pre-incubated for 1 h with 10 μ g/ml cyclohexamide where indicated. Cells were then stimulated for 4h with 100 ng/ml of LPS, and total RNA isolated and analysed for total IL-1ra (top panel) and TNF α (lower panel) mRNA levels. Error bars represent the standard deviation of 3 stimulations. Students t-test p-values of less than 0.01 between the LPS and LPS/cyclohexamide stimulation are indicated by *.

Fig 5 IL-10 secretion reinforces IL-1ra transcription.

A) Wild type BMDMs were stimulated for 3 or 8h with 100 ng/ml LPS. Where indicated an IL-10 neutralising antibody or IgG isotype control antibody was included with the LPS stimulation. Total RNA was then isolated and IL-1ra total mRNA levels determined by Q-PCR as described in the methods. Error bars represent the standard deviation from 3 independent cultures.

B-C) BMDMs were isolated from wild type (black bars) or IL-10 knockout (white bars) mice. Cells were stimulated for the indicated times with 100 ng/ml LPS. Total RNA was then isolated and secretory (B) or cytoplasmic (C) IL-1ra mRNA levels determined by Q-PCR as described in the methods. Error bars represent the standard deviation from 3 independent cultures.

D-F) BMDMs from wild type (black bars) or MSK1/2 knockout (white bars) mice were stimulated with either 100 ng/ml LPS, 100 ng/ml IL-10 or both LPS and IL-10 for either 1 or 3h. Total RNA was isolated and the levels of secretory (D) or cytoplasmic (E) IL-1ra or SOCS3 (F) mRNA determined by Q-PCR. Error bars represent the standard deviation from 3 independent cultures. Students t-test p-values of less than 0.05 between the wild type and knockout samples are indicated by * and less than 0.01 by **.

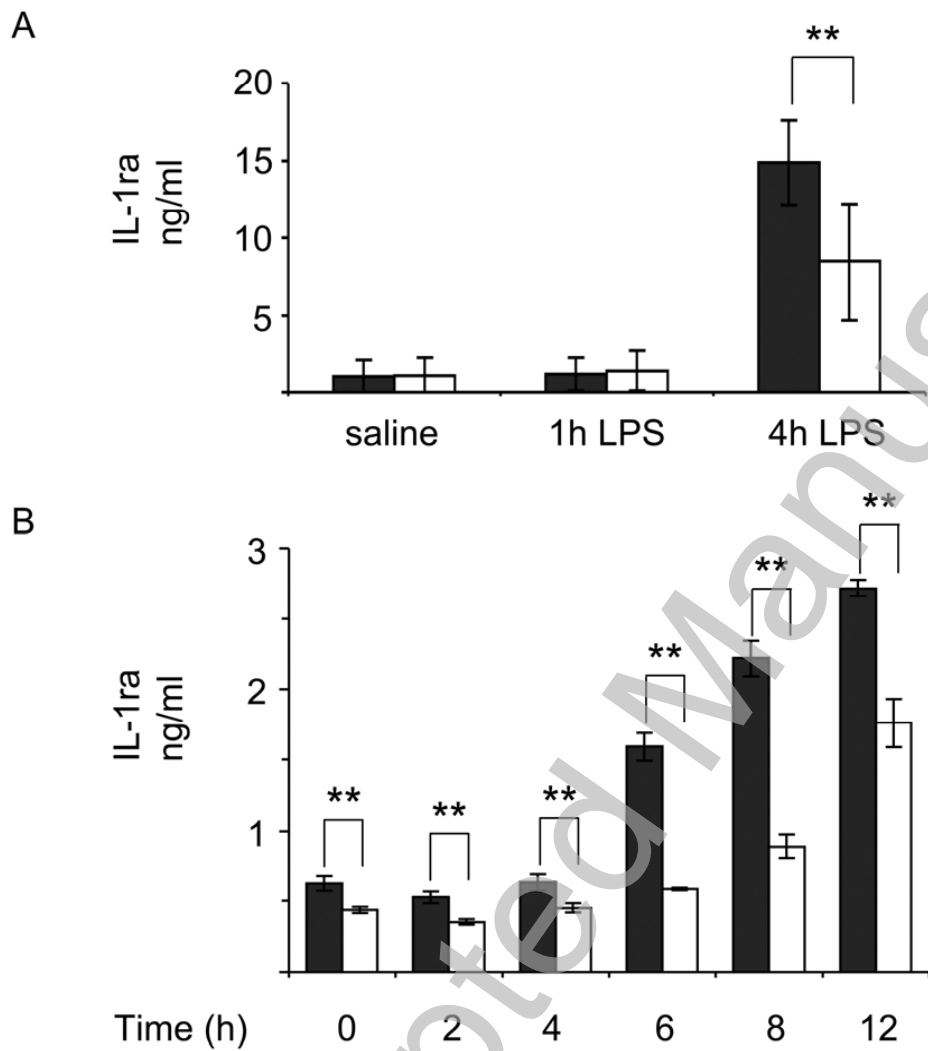
Fig 6 MSK regulates IL-1ra transcription independently of IL-10.

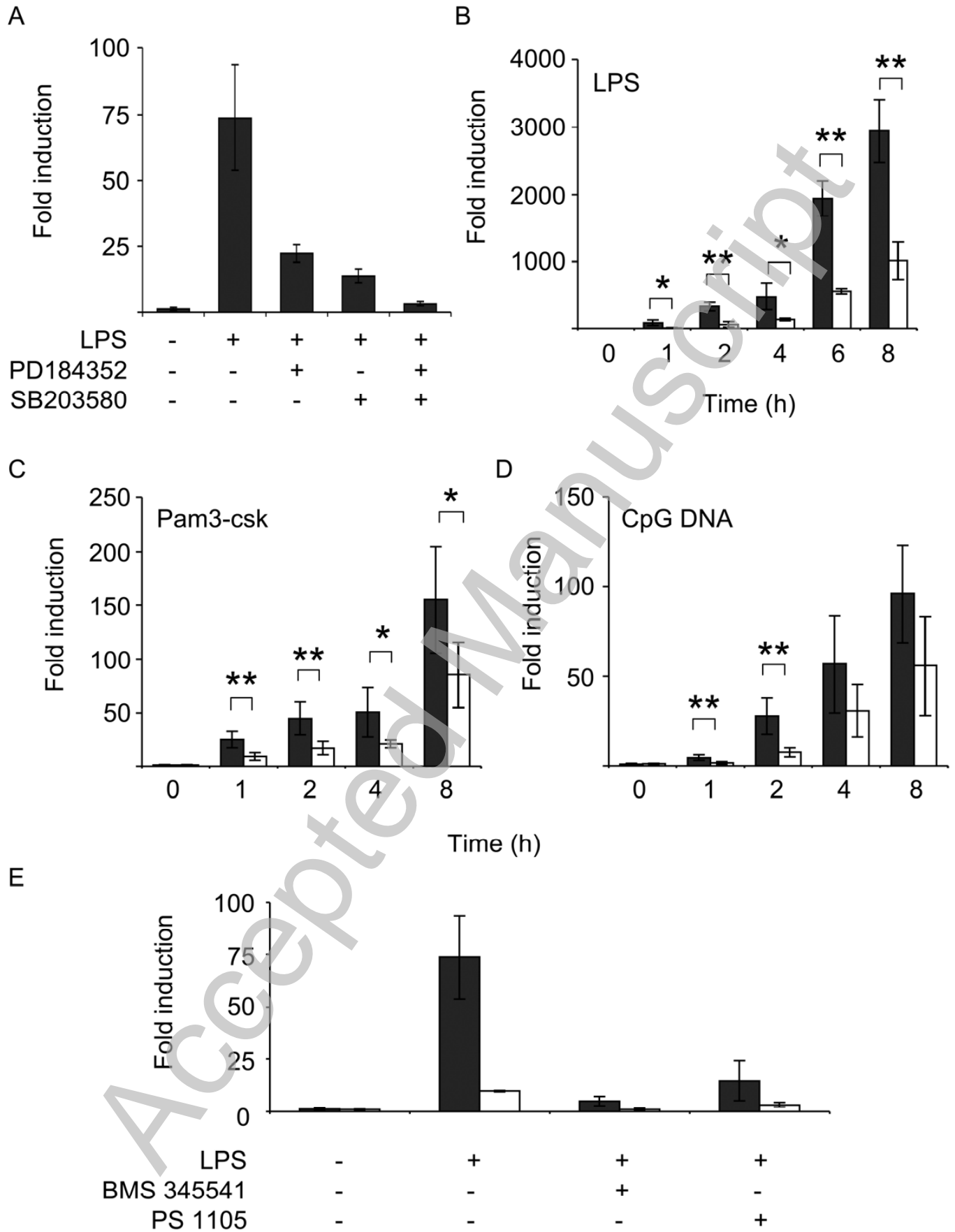
BMDMs were isolated from IL-10 knockout (dark grey bars) or MSK1/2/IL-10 triple knockout (light grey bars) mice. Cells were stimulated for the indicated times with 100 ng/ml LPS and secretory (sec) or cytoplasmic (ic) IL-1ra mRNA induction measured by Q-PCR. Error bars represent the standard deviation of stimulations from 3 independent cultures.

Table I Primer sequences used in Q-PCR.

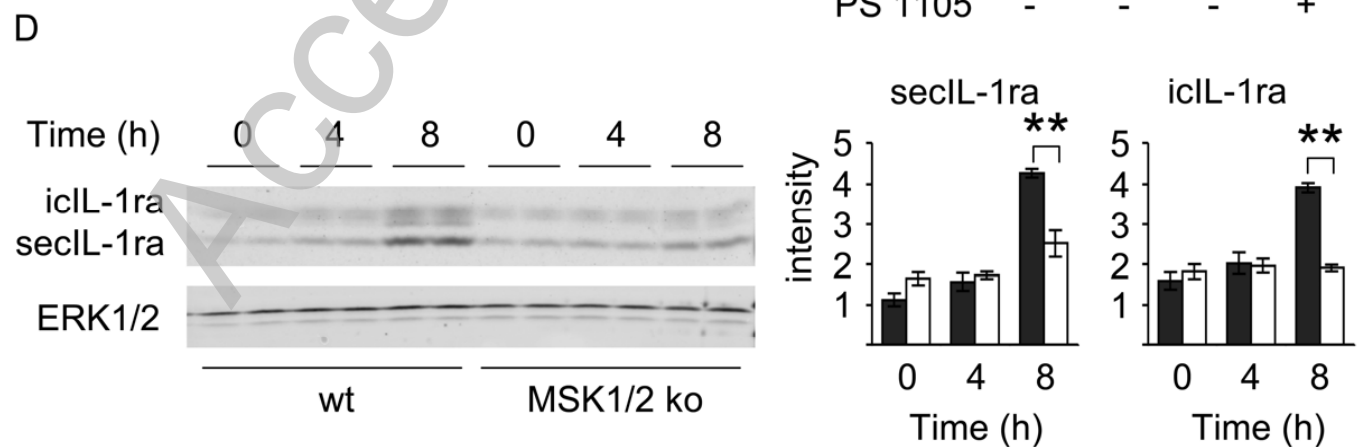
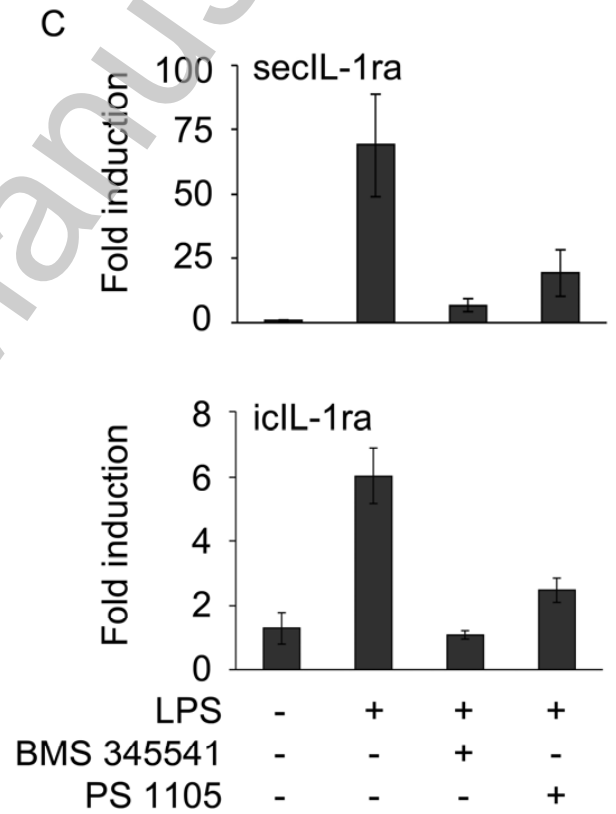
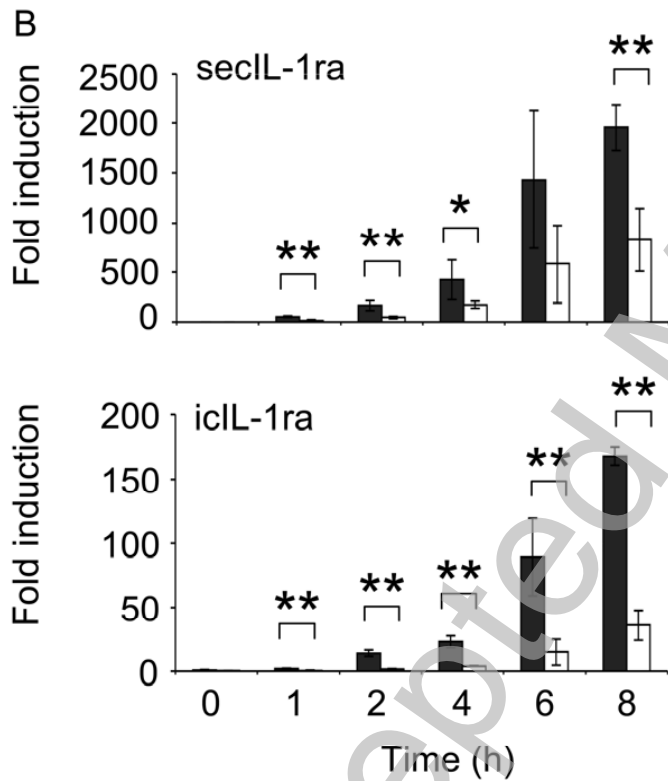
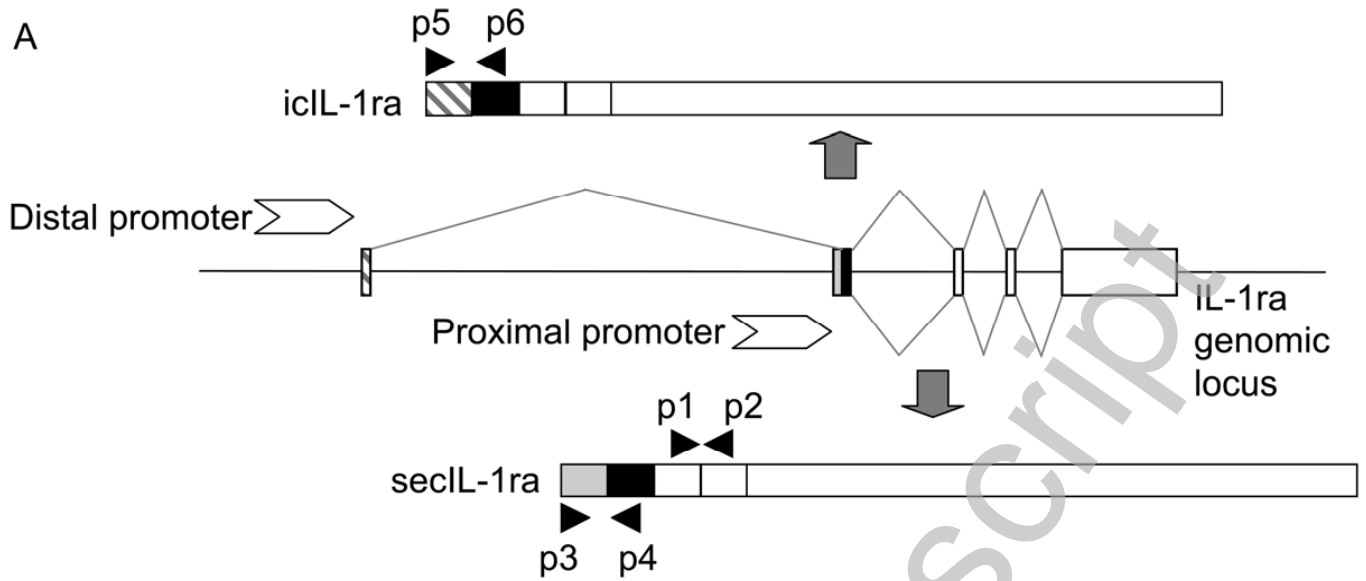
gene	Sense primer	Antisense primer
18s rRNA	GTAACCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
IL-1ra total	TTACAAGGACCAAATATCAAAGTAGAAG	GGATGCCCAAGAACACACTATG
IL-1ra secretory	AGTCGCTAGTCTCTATTGCC	TTCTGAAGGCTTGCATCTTG
IL-1ra cytoplasmic	TCCTTTATACACAGCAAGTCTC	TTCTGAAGGCTTGCATCTTG
Socs3	AGAAGATTCCGCTGGTACTGAG	AAAGATGCTGGAGGGTGGC
TNF	CAGACCCTCACACTCAGATCATC	GGCTACAGGCTTGTCACTCG

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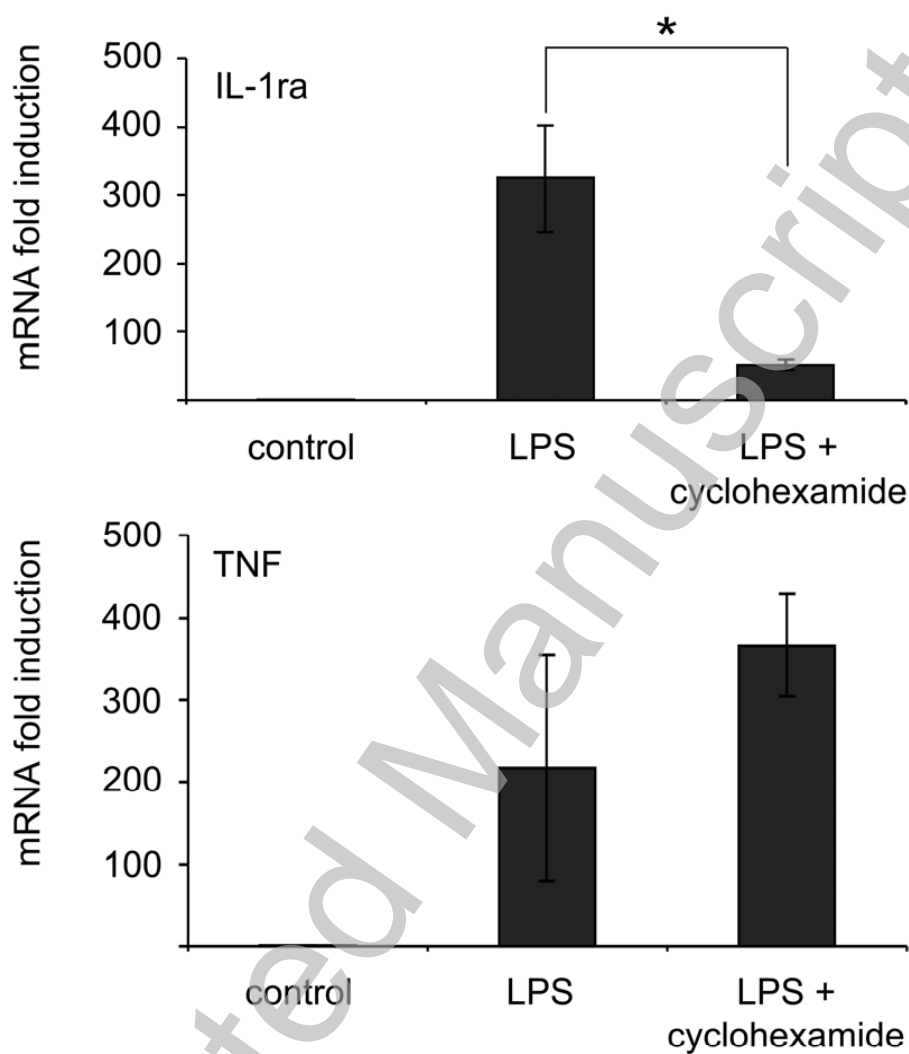


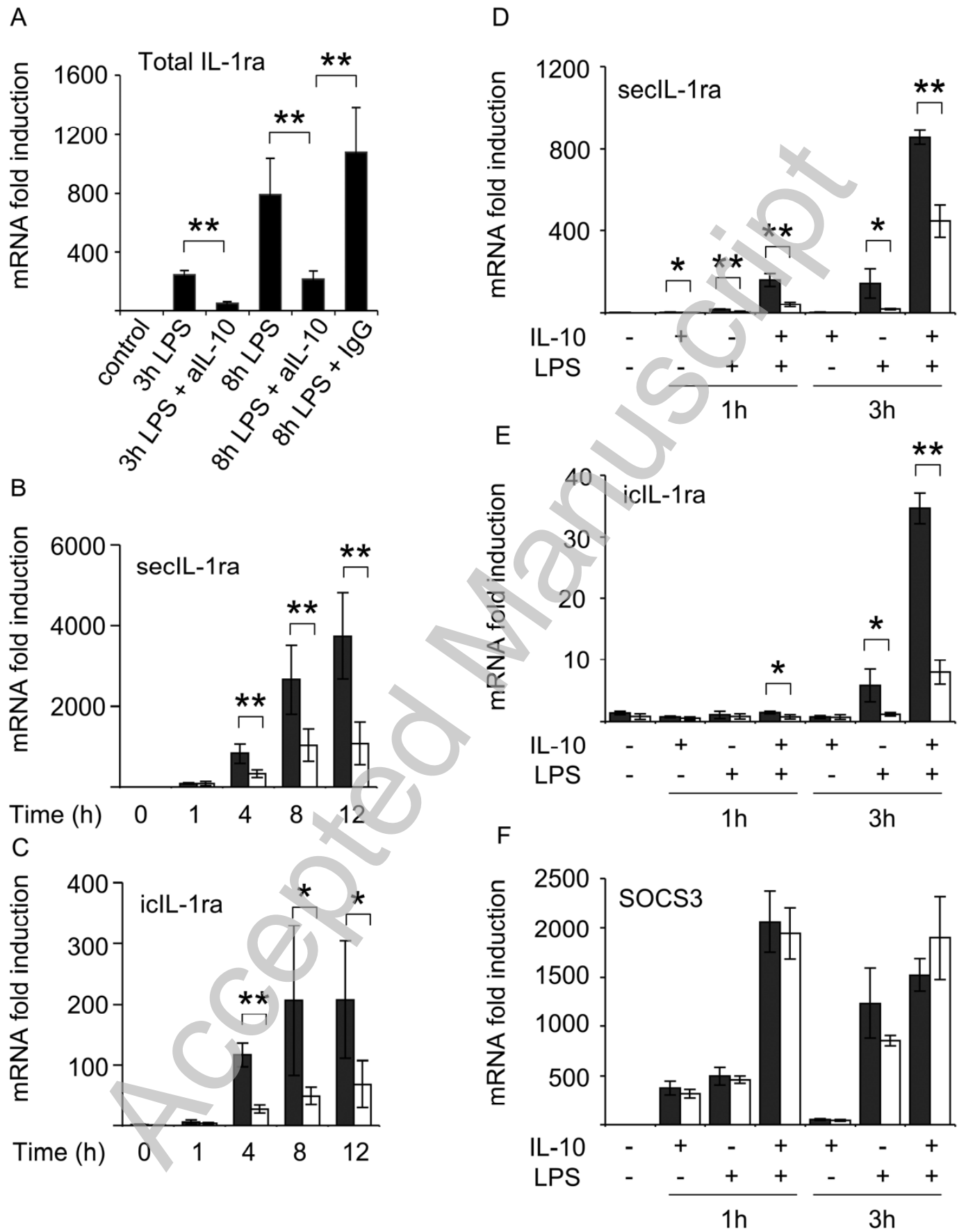


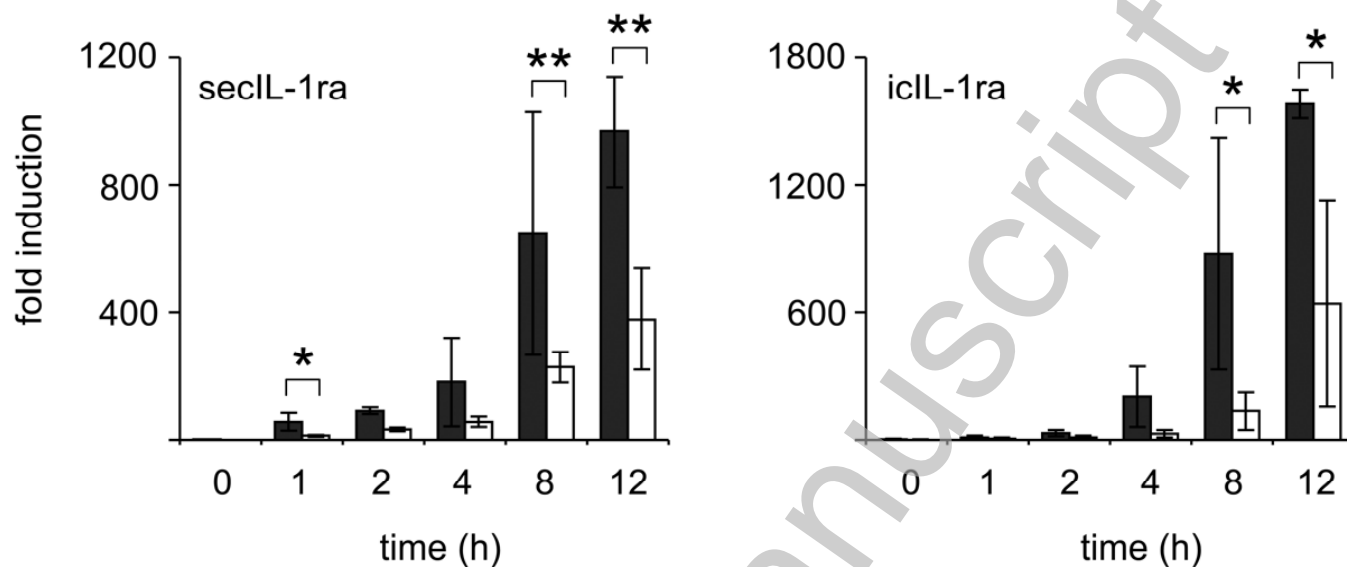
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