Article type : Original Article

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IL-33 regulates cytokine production and neutrophil recruitment via the p38 MAPK-activated kinases MK2/3

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/imcb.12200

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Running title: MK2/3 regulate IL-33 induced mast cell responses

Keywords: Mast cell, p38 MAPK, IL-33, MK2, Myd88, IL-13

ABSTRACT

IL-33 is an IL-1-related cytokine that can act as an alarmin when released from necrotic cells. Once released it can target various immune cells including mast cells, innate lymphoid cells and T cells to elicit a Th2-like immune response. We show here that bone marrow derived mast cells produce IL-13, IL-6, TNF, GM-CSF, CCL3 and CCL4 in response to IL-33 stimulation. Inhibition of the p38 MAPK, or inhibition or knockout of its downstream kinases MK2 and MK3, blocked the production of these cytokines in response to IL-33. The mechanism downstream of MK2/3 was cytokine specific, however MK2 and 3 were able to regulate TNF and GM-CSF mRNA stability. Previous studies in macrophages have shown that MK2 regulates mRNA stability via phosphorylation of the RNA binding protein TTP (Zfp36). The regulation of cytokine production in mast cells was however independent of TTP. MK2/3 were able to phosphorylate the TTP related protein Brf1 (Zfp36l1) in IL-33 stimulated mast cells, suggesting a mechanism by which MK2/3 might control mRNA stability in these cells. In line with its ability to regulate *in vitro* IL-33 stimulated cytokine production, double knockout of MK2 and 3 in mice prevented neutrophil recruitment following intraperitoneal injection of IL-33.

INTRODUCTION

IL-33 is an IL-1-related cytokine that was identified through screening for ligands for the IL-1 receptor family member ST2[1], and it is now established as the major *in vivo* ligand for the ST2 receptor[2-4]. Constitutive IL-33 expression has been observed in non-hematopoietic cells, primarily epithelial and endothelial cells. While IL-1β and IL-18 require cleavage by the inflammasome in order for their secretion and biological activity, this is not true for IL-33. IL-33 lacks a conventional signal peptide and caspase cleavage of IL-33 results in its inactivation[5, 6]. This led to the proposal that IL-33 acts as an alarmin following its release from necrotic cells[7].

The IL-33 receptor comprises of the ST2 (Il1rl1) chain in combination with the IL-1RAcP protein[8]. ST2 expression and IL-33 responsiveness has been reported in a number of cells, notably mast cells[9], type 2 innate lymphoid cells[10-12] and some Th subsets including Tregs and Th2 cells[13-15]. Like other members of the IL-1/TLR receptor superfamily, following ligand binding, the ST2/IL-1RacP dimer is able to recruit the signalling adaptor Myd88[16, 17]. Recruitment of Myd88 promotes the formation of a Myd88osome that includes IRAK4 as well as IRAK1 and/or IRAK2 that is able to activate Traf6[18]. In agreement with this, IL-33 requires Traf6 to activate both the MAPK and NF-κB pathways[19], which in turn promote the production of pro-inflammatory mediators[17, 20, 21]. For example, IL-33 stimulated mast cells have been shown to secrete IL-6, IL-13, TNF, MCP-1 and prostaglandin D2[16, 22-24]. In contrast to IgE receptor mediated mast cell activation, IL-33 stimulation alone does not promote mast cell degranulation[16].

The p38 MAPK family consists of 4 isoforms and acts downstream of cellular stress and inflammatory signals. A role for p38 in the regulation of cytokine production was initially suggested by the finding that a class of pyridinyl imidazoles typified by SB203580, reduced TNF production via inhibition of p38. This led to the development of a large number of p38 inhibitors, most of which target the p38α and β isoforms, although work with gene targeted mice has shown that in macrophages p38α, and not β, is the critical isoform for the regulation of TLR-induced pro-inflammatory cytokine production[18]. p38α is able to activate further downstream kinases, including MKs and MSKs, which can contribute to the ability of p38 to regulate cytokine production[18]. While MK2 and MK3 are solely activated by p38 *in vivo*, MSK1 and the related kinase MSK2 are direct substrates for both p38α and ERK1/2[18]. Work in macrophages has shown that MSKs induce anti-inflammatory feedback pathways and are required for the production of IL-10 by these cells[25, 26]. Knockout of MK2 was found to reduce TNF production in response to TLR agonists both *in vivo* and in isolated macrophages[27]. While MK2 appears to be the more dominant isoform, some compensation does exist between MK2 and MK3, as double knockout of both MK2 and MK3 resulted in a greater suppression of TNF production than knockout of MK2 alone following intraperitoneal injection of LPS in mice[28]. In macrophages, the major mechanism by which MK2 and 3 regulate the production of TNF is via phosphorylation of the mRNA binding protein TTP (also known as Zfp36)[29, 30]. TTP is an mRNA binding protein that recognises AU-rich elements in the 3'UTR of certain mRNAs including that of TNF[31]. Once bound, TTP can both inhibit the translation of the mRNA and promote its degradation. TTP is phosphorylated by MK2 on at least two sites and this inhibits the ability of TTP to repress translation or promote RNA degradation[30, 32, 33]. A critical role for TTP in repressing TNF production has been shown both *in vivo* and in isolated macrophages using TTP knockout mice[34, 35]. Surprisingly, bone marrow derived mast cells from TTP knockout mice showed normal production of TNF and IL-6

in response to LPS[21]. This was attributed to a low basal expression of TTP in mast cells as judged by immunoblotting[21]. Despite this, TTP may still play a role in mast cells under some circumstances; mast cells upregulate TTP mRNA in response to IL-4 stimulation and this has been proposed to explain the repression of IgE induced TNF production by IL-4[36]. Recently, MK2 and 3 have been suggested to play a role in IL-6 and IL-13 induction in IL-33 stimulated mast cells and IL-13 in dendritic cells, however the substrate targeted by MK2 in these cells is not clear[24, 37]. We show here that knockout or inhibition of MK2 and 3 in mast cells blocks TNF, IL-6, IL-13, GM-CSF, CCL3 and CCL4 production in response to IL-33, and that this occurs via both transcriptional and post-transcriptional mechanisms. We also show that the TTP related protein Brf1 is expressed in mast cells and is phosphorylated by MK2/3 in these cells following IL-33 stimulation.

RESULTS

IL-33 induced cytokine production in BMMCs requires p38 MAPK

The IL-33 receptor is part of IL-1 receptor/TLR family of receptor family and acts via Myd88 to stimulate MAPK and NF-κB activation. Consistent with previous reports[38-40], IL-33 was found to activate p38α in bone marrow derived mast cells (BMMCs), as judged by phosphorylation of its TXY activation motif. The activation of p38α was absent in BMMCs from Myd88 knockout mice (Supplementary Figure 1). To examine the roles of p38α in IL-33 induced cytokine induction, BMMCs were treated with the p38α/β inhibitor VX-745 prior to stimulation with IL-33. Pre-treatment with VX-745 did not affect the phosphorylation of p38α on its TXY motif, which is catalyzed by the upstream kinases MKK3 and 6, but did block the phosphorylation of the p38α substrate MK2 (Fig 1a). IL-33 also induced the activation of MSK1, as judged by its phosphorylation on Ser376, a site that correlates to MSK1 activation[41], as well as the phosphorylation of the MSK substrate CREB. VX-745 did not prevent MSK1 or CREB phosphorylation, suggesting that as in other cell types, IL-33 activates MSKs in part via the ERK1/2 pathway. To examine the role of p38α in cytokine induction in BMMCs, cells were stimulated for 8h with IL-33 and the secretion of TNF, IL-6, IL-13 and GM-CSF, as well as the chemokines CCL3 and CCL4, was analysed. IL-33 was able to stimulate the production of the cytokines and chemokines tested, and in each case pre-treatment with VX-745 was able to inhibit their production (Fig 1b).

IL-33 induced cytokine production requires the p38-activated kinases MK2 and MK3

As p38α can mediate its effects via downstream kinases, the role of MSK1/2 or MK2/3 knockout in IL-33 induced cytokine induction was examined. Bone marrow from MSK1/2 double knockout mice was able to differentiate into BMMCs, as judged by similar expression of c-kit and FcεRI and ST2 to wild type BMMCs (Supplementary Figure 2). IL-33 induced phosphorylation of MSK1 and its substrates CREB and ATF1 (Supplementary Figure 3a and b). In line with the ability of both p38 and ERK1/2 to activate MSK1, inhibition of both pathways was required to fully block MSK1 and CREB phosphorylation (Supplementary Figure 3a). As expected, IL-33-stimulated phosphorylation of CREB was lost in MSK1/2 knockout cells (Supplementary Figure 3b). MSK1/2 knockout cells produced similar levels of TNF, IL-6, IL-13 and GM-CSF to wild type cells in response to IL-33 stimulation (Supplementary Figure 3c). In line with the levels of secreted cytokines, the induction of TNF, IL-6, IL-13 and GM-CSF mRNA was similar in wild type and MSK1/2 knockout BMMCs. Although MSK1/2 knockout did not have a major impact on IL-33 induced cytokine induction, it did greatly reduce the induction of nur77 mRNA (Supplementary Figure 3d), an immediate early gene previously shown to be both MSK and CREB dependent in fibroblasts[42, 43].

MK2/3 knockout did not affect the differentiation of BMMCs and both wild type and MK2/3 knockout BMMCs expressed similar levels ST2 (Supplementary Figure 2). Knockout of MK2/3 in BMMCs resulted in a decreased expression of p38α (Fig 2a). This decreased level of p38α is due to a kinase activity-independent role for MK2 in stabilising p38 prior to activation, and is consistent with what has been reported in other cell types in MK2 single or MK2/3 double knockouts[27, 44, 45]. Despite this, p38α activation, as judged by its phosphorylation, following IL-33 stimulation was still apparent, albeit at a lower level than in wild type cells (Fig 2a). MK2/3 knockout BMMCs produced greatly reduced levels of TNF, IL-6, IL-13, GM-CSF, CCL4 and CCL4 following IL-33 stimulation relative to wild type cells (Fig 2b). While this data are consistent with a role for MK2 and 3 in directly regulating cytokine production in BMMCs, the results in the knockout could also be explained by an indirect mechanism due to the decreased levels of p38α in the MK2/3 knockout cells. To resolve this, 3 different MK2/3 inhibitors were used, PF-3644022, Cmp28 and Cmp2s. MK2 and 3 are known to phosphorylate Hsp27[46]. As Hsp27 is not detectable in BMMCs, Cmp28 and Cmp2s were titrated in HeLa cells to determine the concentration required to block Hsp27 phosphorylation in response to anisomycin, a strong activator of the p38 – MK2 pathway[47]. This showed that 5 μM of either compound was able to inhibit MK2/3 in cells (Supplementary Figure 4). Previous studies have shown that 5 μM of PF-3644022 is able to block Hsp27 phosphorylation in HeLa cells[48]. To examine potential off-target activities of Cmp28 and Cmp2s, the compounds were screened *in vitro* against a

panel of 290 kinases. At 1 μM both these compounds inhibited MK2 and MK3 by more than 90%. Cmp2s inhibited PRAK by 72%, but none of the other kinases were inhibited by more than 50%. Cmp28 did not inhibit any of the other kinases tested by more than 25% (Supplementary Figure 4 and Supplementary Table 1). Screening of PF-3644022 showed that it was more potent against MK2 than MK3, and that it showed more off-target activities than Cmp2s and Cmp28 (Supplementary Figure 4 and Supplementary Table 1).

As the stabilisation of p38α by MK2 is independent of the kinase activity of MK2[45], inhibitors of MK2 and 3 would not be expected to affect p38α expression or activation. Consistent with this, treatment of BMMCs with PF-3644022, Cmp28 or Cmp2s did not affect p38 levels or activation in response to IL-33 in BMMCs (Fig 2c). Pre-treatment of BMMCs with PF-3644022, Cmp2s or Cmp28 for 1h before stimulation with IL-33 inhibited the production of TNF, IL-6, IL-13 and GM-CSF (Fig 2d). Together these results are consistent with MK2 and 3 directly regulating cytokine induction downstream of p38α in IL-33 stimulated mast cells. Similar to what was observed in BMMCs, cultured peritoneal mast cells also secreted TNF, IL-6, IL-13 and GM-CSF in response to IL-33 and this was blocked by the MK2 inhibitor Cmp2s (Supplementary Figure 5).

The role of MK2 and 3 has previously been examined in TLR signalling which, like IL-33, can act via Myd88-dependent signalling. This work has focused predominantly in macrophages and dendritic cells where MK2 is required for maximal TNF production[28]. It is less clear if loss of MK2 and 3 results in a general block in cytokine induction in these cells as was seen in BMMCs (Fig 2). To determine if this was due to a difference between IL-33 and TLR agonists or a mast cell specific effect, cytokine induction in LPS-stimulated MK2/3 knockout mast cells and bone marrow derived macrophages (BMDMs) was examined. LPS was used as IL-33 did not induce cytokine production in BMDMs (data not shown). BMDMs produced TNF, IL-6, IL-12p70 and IL-12p40 in response to LPS. MK2/3 knockout BMDMs produced lower amounts of TNF than wild type cells in response to LPS, however IL-6, IL-12p70 and IL-12p40 production was not reduced (Supplementary figure 6a). BMDMs were obtained via culture of bone marrow cells in M-CSF. Differentiation of bone marrow cells with GM-CSF gives rise to a heterogeneous macrophage / dendritic cell population[49]. LPS-induced TNF secretion was decreased in MK2/3 knockout cells relative to wild type cells in GM-CSF differentiated cultures while only minor effects were seen on IL-6, IL-13 and IL-12p40 secretion (Supplementary figure 6b). To examine the role of MK2/3 in cytokine production in TLR-stimulated mast cells, BMMCs were stimulated with TLR4 agonist LPS. Similar to IL-33, LPS was able to stimulate TNF, IL-6, IL-13 and GM-CSF production in BMMCs, and in each case the production of these cytokines was reduced by MK2/3 knockout (Supplementary figure 6c). In line with this, LPS induced cytokine

production in BMMCs was inhibited by the MK2/3 inhibitors PF-3604422 and Cmp2s (Supplementary figure 7).

In macrophages, MK2 regulates TNF production via the phosphorylation of TTP, a protein that binds to AU-rich elements in the 3'UTR of a subset on mRNAs resulting in destabilisation of the mRNA[31, 50]. To determine if MK2/3 mediated TTP phosphorylation could explain the effects of MK2/3 knockout in mast cells, BMMCs were derived from TTP knockout bone marrow. Loss of TTP did not have a major effect on the IL-33 induced secretion of TNF, IL-6, IL-13 or GM-CSF (Fig 3a). To confirm these findings, BMMCs were also generated from mice carrying alanine mutations at the two major MK2 phosphorylation sites, Ser52 and Ser178, in TTP[30]. Again, the IL-33 induced secretion of TNF, IL-6, IL-13 and GM-CSF was similar in TTP knockin and wild type BMMCs. Furthermore, pre-treatment of the knockin cells with either PF-3604422, Cmp2s or Cmp28 was able to inhibit cytokine induction, indicating that MK2 and 3 could act independently of TTP phosphorylation (Fig 3b).

The p38-MK2/3 pathway regulates IL-33 induced translation of TNF

To examine the mechanism by which MK2/3 regulates IL-33 induced cytokine production, the effects of MK2/3 knockout on cytokine mRNA induction was examined. IL-33 stimulation increased the mRNA levels for TNF, IL-6, GM-CSF and IL-13 in wild type BMMCs. Loss of MK2 and 3 did not greatly affect the induction of either TNF mRNA or the unspliced primary TNF transcripts relative to wild type cells (Fig 4a, b). To examine the upregulation of TNF protein, cells were stimulated with IL-33 in the presence of agents that block secretion and then intracellular levels of TNF determined by flow cytometry. This showed that, unlike wild type cells, MK2/3 knockout BMMC failed to upregulate TNF protein in response to IL-33 (Fig 4c). Similar effects on TNF protein induction were seen by pre-treating wild type cells with the MK2 inhibitor PF-3644022 (Fig 4d). MK2/3 knockout BMMCs showed reduced mRNA induction of IL-6 and IL-13 in response to IL-33 relative to wild type cells, with the maximal effect seen around 1h of stimulation. This correlated to a lower induction of the primary transcript for IL-13, and to a lesser extent IL-6, in MK2/3 knockout cells at this time point (Fig 4a, b). For GM-CSF, the initial induction of mRNA was normal in the MK2/3 knockouts however levels were lower in MK2/3 knockouts compared to wild type BMMCs at 8h of LPS stimulation (Fig 4a).

To determine if MK2/3 knockout might affect cytokine RNA stability, cells were stimulated for 1h with IL-33 and further transcription blocked by the addition of DRB (5,6-dichloro-1*ϐ*-1-ribofuranosylbenzimidazole) and actinomycin D and the decay of mRNA levels measured over time.

TNF mRNA was unstable and decayed with a half-life of approximately 55 min in wild type cells (Fig 5a). In MK2/3 knockout BMMCs TNF mRNA was less stable with a half-life of approximately 30 min. In wild type cells IL-6, IL-13 and GM-CSF mRNAs were found to be stable (half-life >2h). Knockout of MK2/3 did not have a major effect on the stability of IL-6 or IL-13 mRNA however MK2/3 knockout did decrease the stability of the GM-CSF mRNA (Fig 5a). Similar results were observed when cells were stimulated for 3h with IL-33 before the addition of DRB and actinomycin D (data not shown).

MK2/3 phosphorylate Brf1 in response to IL-33

While the effects of MK2/3 on TNF and GM-CSF production BMMCs are TTP-independent, MK2 has been reported to phosphorylate Brf1, a TTP-related protein, *in vitro* and in the fibrosarcoma cell line, HT1080[51]. Brf1 protein levels were increased in IL-33 stimulated BMMCs (Fig 5a), as were mRNA levels for both Brf1 and Brf2 (Supplementary figure 8). Multiple bands were observed in the immunoblots, consistent with the multiple phosphorylation sites known to occur on Brf1 affecting its electrophoretic mobility on SDS-polyacrylamide gels. Knockout of MK2 and 3 resulted in a decreased phosphorylation of Brf1 as judged by the decrease in the upper band in the Brf1 blots from MK2/3 knockout compared to wild type BMMCs. Similar results were observed when cells were treated with the MK2/3 inhibitor cmp2s (Fig 5c). Brf1 has been reported to be phosphorylated on several sites including Ser92. Blotting with a phospho-specific antibody for this site showed that MK2/3 knockout or MK2/3 inhibitor reduced the IL-33 induced phosphorylation of Brf1 on this site in mast cells (Fig 5b, c). Phosphorylation of Brf1 has been reported to enable it to interact with 14-3-3 proteins[52, 53]. In line with this, more Brf1 was observed in 14-3-3 pull downs from lysates from IL-33 stimulated BMMCs relative to unstimulated cells. This was blocked by knockout of MK2/3 (Fig 5d) or pre-treatment of wild type cells with an MK2/3 inhibitor (Fig 5e). To confirm that the Brf1 in the 14-3-3 pulldowns was phosphorylated, 14-3-3 pull downs were treated with λ phosphatase and then analysed by immunoblotting. This showed that the Brf1 in the phosphatase treated samples ran at a lower molecular weight, as would be expected with the removal of phosphate groups (Supplementary figure 9). Blotting of the pull downs with an antibody that recognizes the phosphorylation of Brf1 on Ser92 also indicated that Brf1 was phosphorylated in the pulldowns and that this phosphorylation can be removed by the phosphatase treatment (Supplementary figure 9).

PI3K signalling is required for maximal IL-33 induced cytokine production

In addition to being phosphorylated by MK2 and/or 3, Brf1 has also been reported as a substrate for Akt[52, 53]. A role for MK2 and 3 in the activation of Akt has previously been found in TLR stimulated macrophages[48]. To determine if Akt might play a role in regulating IL-33-induced cytokine production, the Akt inhibitors Akti 1/2 and MK-2206 as well as the PI3 kinase inhibitors GDC-0941 and PI-103 were used. All 4 inhibitors reduced the induction of TNF, IL-6, IL-13 and GM-CSF (Fig 6a). Analysis of cytokine mRNA induction showed that inhibition of PI3 Kinase or Akt reduced the induction of TNF and IL-13 mRNA in response to IL-33 (Fig 6b). GM-CSF mRNA was not significantly affected ($p>0.05$) by PI3 Kinase inhibitors, although some reduction was seen in the presence of Akt inhibitors. IL-6 mRNA levels were not affected by either PI3 Kinase or Akt inhibitors following 1h of IL-33 stimulation (Fig 6b). While both MK2/3 and Akt are involved in IL-33 induced cytokine induction, the smaller inhibition seen by blocking PI3 Kinase compared to MK2/3 indicate that the effects of MK2/3 knockout cannot be fully explained by cross talk between the MK2/3 and Akt pathways. To confirm this, MK2/3 inhibitors were added in combination with a PI3 kinase inhibitor. As in Fig 6a, GDC-0941 and PI-103 reduced IL-33 induced TNF, IL-6, IL-13 and GM-CSF production. The remaining cytokine production in the presence of PI3 kinase inhibitors could however be further reduced by the addition of the MK2 inhibitor PF-3604422 (Fig 6c). Finally, we tested if the phosphorylation of Akt on Thr308 and Ser473 required MK2/3 in IL-33 stimulated BMMCs. Both residues were phosphorylated in response to IL-33 in wild type cells. In MK2/3 knockout cells the phosphorylation of Akt on both Thr308 and Ser473 was reduced following 30min of stimulation, however at 60min after IL-33 stimulation Akt phosphorylation on Ser473 was slightly enhanced in the MK2/3 knockout relative to wild type cells (Fig 6d). Similar effects were observed with the p38 inhibitor VX-745 (Fig 6e). This would indicate that p38 – MK2/3 signaling regulates the kinetics of Akt activation, but is not completely essential for IL-33 induced Akt activation.

MK2 and 3 regulate IL-33 induced neutrophil recruitment *in vivo*

Intraperitoneal injection of IL-33 in mice has previously been shown to promote neutrophil recruitment in wild type but not $W^{sh/sh}$ mice, which lack mature mast cells[54]. We therefore tested the effect of IL-33 injection in MK2/3 double knockout mice (Fig 7a). Control experiments using LPS demonstrated that MK2/3 knockout mice recruited more neutrophils compared to wild type animals, arguing against an essential role for MK2/3 in neutrophil recruitment. In contrast, IL-33 injection recruited much fewer neutrophils in MK2/3 knockout mice relative to wild type controls

(Fig 7a). Knockout of MSK1 and 2 did not affect IL-33-induced neutrophil recruitment in this model (Fig 7a) consistent with the minor effects of MSKs on IL-33 induced cytokine production in isolated mast cells (Supplementary Figure 2). To confirm that loss of MK2/3 in mast cells was able to affect the ability of these cells to secrete chemoattractant for neutrophils, peritoneal mast cells were cultured from wild type and MK2/3 knockout mice. These cells were then used in Transwell assays to examine their ability to promote the migration of wild type neutrophils. The presence of mast cells in the lower chamber was able to stimulate a low level of neutrophil migration. Stimulation of the wild type mast cells with IL-33 resulted in an increased neutrophil migration. IL-33 stimulation of MK2/3 knockout cells resulted in an increase in neutrophil migration, but this was lower than seen with wild type mast cells (Fig 7b). IL-33 stimulated the production of the chemokines CCL3 and CCL4 by peritoneal mast cells, and this was reduced by knockout of MK2 and 3 (Fig 7c). CCL3 has previously been reported to be a relatively poor chemoattractant for neutrophils, but its ability to promote neutrophil recruitment is increased when the neutrophils are stimulated with GM-CSF[55]. In line with this we observed a greater attraction of neutrophils to a combination of CCL3 and GM-CSF than CCL3 alone in Transwell assays (Supplementary figure 10). CXCL2 was also detected in the media from peritoneal mast cells. While the levels were not increased by IL-33 treatment, lower levels were detected from MK2/3 knockout than wild type mast cells (Fig 7c).

DISCUSSION

p38α has an established role in regulating cytokine production downstream of TLR signalling in macrophages and dendritic cells. With the exception of TLR3, TLRs can stimulate p38 activity via Myd88, a pathway shared with IL-1 receptor family members including the ST2/IL-1RAcP IL-33 receptor. We show here that treatment of BMMCs with the p38α/β inhibitor VX-745 greatly inhibited the IL-33 induced production of TNF, IL-6, IL-13, GM-CSF, CCL3 and CCL4. This is in agreement with previous studies have also shown that p38 inhibitors can block IL-33 induced TNF, IL-6 and IL-13 production in BMMCs[38, 39]. p38α regulates cell function in part via the activation of two groups of downstream kinases, MK2/3 and MSK1/2, both of which impact on TLR-induced cytokine production in macrophages[18]. Both MK2/3 and MSK1/2 were activated in response to IL-33 in BMMCs (Fig 1). In the case of MSK1/2, inhibition of both the p38α and ERK1/2 MAPK pathways was required to block IL-33 induced MSK1 activation and phosphorylation of the MSK substrate CREB (Supplementary figure 3), a finding consistent with MSK activation in response to TLR stimulated macrophages[25]. MSK1/2 knockout did not have a major effect on cytokine production in IL-33 stimulated BMMCs. While MSKs are known to regulate Myd88-dependent cytokine induction

in macrophages and dendritic cells, they are not required for TLR induced IL-10 production in B cells[25, 56, 57]. The role of MSKs in cytokine induction therefore seems to be cell type specific.

In contrast to MSK1/2, MK2/3 knockout had a much greater impact on cytokine production in IL-33 stimulated BMMCs. MK2/3 knockout greatly reduced TNF, IL-6, IL-13, GM-CSF, CCL3 and CCL4 secretion in response to IL-33 in BMMCs (Fig 2), a finding consistent with a recent report showing MK2/3 knockout reduced IL-33 stimulated IL-6 and IL-13 production in BMMCs[40]. MK2/3 knockout reduces p38α levels due to a non-catalytic role for MK2 in stabilizing p38α. The results from the MK2/3 knockout cells could be interpreted as either being due to a loss of p38α or a direct role for MK2/3 catalytic activity in regulating cytokine induction. We therefore examined the effects of 3 MK2/3 inhibitors – these did not affect p38α expression levels but did replicate the effects of MK2/3 knockout on cytokine production (Fig 2).

In both macrophages and dendritic cells MK2 is required for maximal TNF production[28], however in these cell types MK2 and 3 are not essential for the production of all cytokines. For example MK2 knockout reduced IL-6 production in macrophages on prolonged LPS stimulation but initial IL-6 production was not reduced[58], while in dendritic cells MK2 knockout did not reduce IL-6 or IL-12 production[59]. In agreement with these reports we found that MK2/3 knockout did not prevent LPS-induced IL-6, IL-13 or IL-12p40 production in BMDMs or GM-CSF differentiated bone marrow cells. The reason for the more extensive role of MK2 and 3 in mast cells is not clear, however our data would suggest that MK2 and 3 regulate cytokines in mast cells via different mechanisms than they use in macrophages.

The regulation of cytokine production by MK2 and 3 in mast cells is likely to involve regulation of both transcription and mRNA stability, and relative contribution may be cytokine specific. For both IL-6 and IL-13, MK2/3 knockout reduced the induction of the primary transcript, suggesting a role for MK2/3 in the transcription of these genes. In contrast, for TNF and GM-CSF MK2/3 knockout did not have a negative effect on induction of the primary transcript but did affect the stability of the mRNA suggesting that MK2/3 regulate these genes via a post transcriptional mechanism. The post transcriptional regulation of cytokines by MK2/3 in macrophages has been shown to involve the protein TTP, which regulates stability and/or translation of AU-rich element containing mRNAs, including TNF[40]. TTP however did not contribute to the regulation of cytokine production in IL-33 stimulated BMMCs. Neither TTP knockout nor Ser to Ala knockin mutation of the 2 major MK2 phosphorylation sites in TTP affected IL-33 stimulated production of TNF, IL-6, IL-13 or GM-CSF in BMMCs (Fig 3). MK2/3 did however regulate mRNA stability in IL-33 stimulated BMMCs; TNF, and GM-CSF mRNA stability was reduced in MK2/3 knockout BMMCs (Fig 5).

Brf1 (Zfp36l1) and Brf2 (Zfp36l2) are related to TTP, and like TTP, are known to regulate mRNA stability[26, 50, 52, 53]. The role of Brf1 and 2 in immunity however has not been studied as extensively as that of TTP. Brf1 and Brf2 are involved in early B cell development where they regulate senescence to enable recombination at the immunoglobulin locus[60] while in T cell development they are involved in the β-selection checkpoint, and loss of Brf1 and 2 results in leukemia[61]. The roles of Brf1 and 2 in innate immune cells are not well understood. Brf1 can be induced by inflammatory stimuli in macrophages, however Brf1 knockout, unlike TTP knockout, does not affect TNF or IL-6 induction in these cells[62]. Immunoblotting showed that BMMCs expressed Brf1 and that MK2/3 knockout or inhibition reduced Brf1 phosphorylation on Ser92. Two additional sites exist in Brf1 that conform to the MK2 consensus sequence, and it is possible that these sites are also phosphorylated by MK2/3 in IL-33 stimulated mast cells. The lack of phospho-specific antibodies to these sites means we were not able to address this directly. The MK2 consensus sequence (ϕ-X-R-X-X-pS/T) is similar to that required for binding to 14-3-3 proteins. Following IL-33 stimulation, Brf1 was able to bind to 14-3-3 proteins and this was blocked by MK2/3 inhibitors or MK2/3 knockout. It is therefore possible that MK2/3 regulate cytokine stability downstream of IL-33 via Brf1 phosphorylation resulting in Brf1 being sequestered by 14-3-3 proteins.

In addition to regulating TTP in TLR stimulated macrophages, MK2/3 also contribute to the activation of the PI3 Kinase - Akt pathway. While the molecular mechanism behind this is unclear, in macrophages MK2/3 were found to act at the level of PIP3 production in the membrane, either via promoting PI3 kinase activity or inhibition of PIP3 phosphatases[48]. We found that in BMMCs, IL-33 was able to promote activation of Akt as judged by phosphorylation of Akt on Ser473 and Thr308 (Fig 6). Drube *et al*. have also recently reported that IL-33 can induce Ser473 phosphorylation in Akt, although Thr308, which is essential for Akt activation, was not addressed in this study[40]. Drube *et al*. also reported that MK2/3 knockout abolished IL-33 induced Akt Ser473 phosphorylation and suggested that this contributed to the ability of MK2 and 3 to regulate IL-6 and IL-13 production. In contrast, we did not see complete inhibition of Ser473 or Thr308 phosphorylation by MK2/3 knockout or p38 inhibition, although MK2/3 were more important in regulating the kinetics of Akt phosphorylation in response to IL-33, with the initial phosphorylation of Akt being slower in the MK2/3 knockouts. The reason for this difference is not clear. Interestingly the remaining cytokine induction in the presence of PI3 kinase or Akt inhibitors was still sensitive to MK2 inhibitors, indicating that in our experiments MK2/3 could regulate cytokine induction via a PI3 kinase - Akt independent mechanism.

MK2/3 knockout reduced neutrophil recruitment to the peritoneal cavity following i.p. injection of IL-33. Previous studies using this model have found that neutrophil recruitment did not occur in W^{sh}/W^{sh} mice, which lack mast cells. This could be rescued by injection into the peritoneal cavity of wild type BMMCs but not BMMCs deficient in the IL-33 receptor[63]. *In vivo*, neutrophil recruitment is tightly regulated and may involve waves of different factors, including CXCL1/2, CCL3/4 and leukotriene B4 (LTB4)[64, 65]. The major chemoattractant for neutrophils in the i.p. IL-33 model is not known. In response to intraperitoneal injection of LPS, neutrophil recruitment can be reduced by antibodies blocking CXCL1 or CXCL2, however a combination of both antibodies did not completely block neutrophil recruitment consistent with the involvement of other factors[63]. Neutralising antibodies against CXCL1 alone had no effect on neutrophil recruitment following i.p. injection of IL-33[54]. LPS could activate both macrophages and mast cells in the peritoneal cavity, and thus both cells types could contribute to neutrophil recruitment in this model. Of note MK2/3 knockout did not decrease neutrophil recruitment in response to LPS, which is consistent with the reduced dependence on MK2 and 3 for the production of pro-inflammatory cytokines in macrophages relative to mast cells (Supplementary figure 6). In contrast, mast cells comprise the major ST2+ve cell population in the peritoneal wash. The low numbers of mast cells present however makes direct measurement of chemokines in the peritoneal wash following IL-33 injection problematic and we failed to detect cytokines in the peritoneal wash following IL-33 injection, most probably due to the dilution during the washing procedure (data not shown). In BMMC cultures IL-33 strongly induced the mRNA for CCL3 and CCL4. mRNA for CXCL1 and CXCL2 was also induced, but the fold induction and relative level of these mRNAs was less than for CCL3 and CCL4 (Supplementary figure 10). Isolated peritoneal mast cells produced CCL3 and CCL4 in response to IL-33, and this was reduced in MK2/3 knockouts relative to wild type mast cells (Fig 7c). CXCL2 was also detected in the media, but little stimulation was seen in response to IL-33. As peritoneal mast cells did not produce high levels of the classical neutrophil chemokines CXCL1 and 2, it is possible that neutrophil recruitment in this model may require the production of multiple factors. TNF knockout BMMCs were reported to be less effective than wild type BMMCs at rescuing neutrophil recruitment in the W^{sh}/W^{sh} mice[54]. GM-CSF, another MK2/3 dependent cytokine produced by mast cells, may also play a role in priming neutrophils; while CCL3 is a poor chemoattractant for naïve neutrophils *in vitro*, GM-CSF is reported to prime neutrophils to make them sensitive to CCL3 as a chemoattractant[55]. Further studies will be required to address these questions. Our data would also not exclude a role for MK2/3 in other cell types *in vivo*. Type II innate lymphoid cells also express the IL-33 receptor and are present in the mesenteric membranes[13]. It is possible that IL-33 also induced chemokines in these cells *in vivo* and that this could contribute to the neutrophil recruitment.

Together these studies show that MK2 and 3 are critical for regulating cytokine production in IL-33 or LPS stimulated mast cells. The way in which MK2/3 regulate these processes is cytokine specific and may occur via transcriptional or post-transcriptional mechanisms depending on the cytokine. The roles of MK2 and 3 in IL-33 stimulated mast cells appear more extensive than those in TLR-stimulated macrophages. IL-33 also acts on ILC2s and T cell subsets, it will therefore be interesting to determine how MK2 and 3 affect IL-33 responses in these cells.

METHODS

Mice

TTP knockout, TTP S52A / S178A double knockin, MK2/3 double knockout, MSK1/2 double knockout and Myd88 knockout mice have been described previously and had been backcrossed onto C57Bl/6 for at least 12 generations[27, 30, 34, 44, 47, 66]. Mice were housed in individually ventilated cages and allowed free access to food and water. Colonies were maintained under specific pathogen-free conditions and work was approved via local ethical review and carried out subject to a UK Home Office licence.

Cell culture

Bone marrow derived mast cells (BMMCs) were derived from adult mice. Mice were sacrificed and femurs excised under sterile conditions. The bone marrow was flushed from the femurs with sterile PBS and the bone marrow suspension was passed through a 100 µm cell strainer (Greiner bio-one, Stonehouse, UK). Cells were centrifuged at 1,000 rpm for 5 minutes, and the pellet was resuspended in 25 ml mast cell media (RPMI 1640 medium containing 10% FBS (Biosera/Labtech, Heathfield, UK), 5mM L-Glutamine (GIBCO Life Technologies), 100 U mL^{-1} Penicillin (GIBCO Life Technologies), 100 µg mL^{-1} Streptomycin (GIBCO Life Technologies, Fisher Scientific, Loughborough, UK), 25 mM HEPES (Lonza, Edinburgh, UK), 1 mM sodium pyruvate (Lonza), 1X non-essential amino acids (Lonza), 50 µM 2-mercaptoethanol and 30 ng mL^{-1} IL-3 (PeproTech. London, UK)) and transferred to a 75 cm^2 tissue culture flask. The cells in suspension were cultured for 5-6 weeks at 37^{o}C and 5% CO_2, and were split twice a week to refresh the media and maintain their density at approximately $1x10^6$ cells mL^{-1}. For measurement of cytokine production, cells were suspended in fresh media and then rested for 48h before stimulation with IL-33.

To culture peritoneal mast cells from mice, the peritoneal cavity was washed with PBS with 2mM EDTA and 0.5% BSA. Cells were pelleted by centrifugation and resuspended in RPMI 1640 supplemented with 10% FBS (Biosera/Labtech), 5 mM L-Glutamine (GIBCO Life Technologies), 100 UmL^{-1} Penicillin (GIBCO Life Technologies), 100 $\mu g\ mL^{-1}$ Streptomycin (GIBCO Life Technologies), 25 mM HEPES (Lonza), 1 mM sodium pyruvate (Lonza), 1X non-essential amino acids (Lonza), 50 µM 2-mercaptoethanol, 20 ng mL^{-1} SCF (PeproTech) and 30 ng mL^{-1} IL-3 (PeproTech) and incubated at 37^{o}C and 5% CO_2. After 24 hours non-adherent cells were discarded and the media replaced. Thereafter both suspension and adherent cells were passaged into fresh media every 3 days and the cells used for experiments at 14 days. Analysis of the cells by flow cytometry showed that at 14 days they were >94% positive of c-kit and FcεR and expressed the ST2 receptor.

BMDMs were cultured as described previously while GM-CSF bone marrow differentiated cells were cultured as for BMDMs except that the M-CSF in the culture media was replaced with 5ng mL^{-1} GM-CSF (PeproTech)[67].

Unless otherwise indicated, kinase inhibitors were dissolved in DMSO and used at the following final concentrations: VX-745 (Selleck, Stratech, Ely, UK), 1 µM; PI-103 (Merck Millipore, Watford, UK), 1 µM; GDC-0941 (Axon Medichem, Groningen, Netherlands), 1 µM; Akti 1/2 (Merck Millipore), 1 µM; MK2206 (Selleck), 1 µM; PF-364402 (Tocris, Bio-Techne, Abingdon, UK), 5 µM; Cmp28 (compound 28 in[68, 69], synthesized in house), 5 µM; Cmp2s (Compound 2s in[68, 69], synthesized in house), 5 µM. Synthesis of Cmp28 and Cmp2s was based on previous publications[68, 69]. *In vitro* kinase screening data for Cmp28 and Cmp2s was carried out by Merck Millipore and for PF 364402 by the MRC International Centre for Kinase Profiling (Dundee, http://www.kinase-screen.mrc.ac.uk). Cells were stimulated as indicated in the legends using 10 ng mL^{-1} murine IL-33 (PeproTech) or 100 ng mL^{-1} LPS (*Escherichia coli* strain O26:B6; Sigma; L2654). For the 0 time stimulations in the figures, cells did not receive any IL-33 or LPS. The inhibitors did not compromise the viability of the mast cells at the concentrations and times used in this study (Supplementary figure 11). IL-33 and GM-CSF were from PeproTech and CCL3 was from Biolegend UK (London).

Flow cytometry of mast cells

Cells were pelleted and resuspended in PBS containing 1% FCS and incubated with FcBlock (1:50, BD Biosciences, San Jose, US) for 10 min and then stained with anti-c-Kit-FITC (1 in 500, BD Biosciences,

#553354), anti-FcεR1-PE (1 in 400, eBiosciences VWR International Ltd Lutterworth, UK, #12-5898-81) and anti-ST2-BV421 (1 in 400, BioLegend #145309) for 30 min. Cells were then washed twice and analysed by flow cytometry. For analysis, live cells were identified by gating based on forward and side scatter.

Immunoblotting

Cells were lysed in triton lysis buffer: 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.27 M sucrose, 1% (vol/vol) Triton X-100, 0.1% (vol/vol) 2-mercaptoethanol, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 1 mM PMSF. Lysates were clarified by centrifugation (13,000 rpm for 10 min at 4°C) and supernatants snap-frozen and stored at −80°C. Protein concentration was determined with Coomassie Protein Assay Reagent (Fisher Scientific, Loughborough, UK). Proteins were separated on 10% polyacrylamide gels and immunoblotting carried out using standard techniques. Antibodies recognizing phospho-Thr180/Tyr182 p38 (#4511), total p38 (#9212), total ERK1/2 (#9102), phospho-Ser473 Akt (#9271), phospho-Thr308 Akt (#9275), BRF1/2 (#2119), phospho-Ser376 MSK1 (#9591), phospho-Ser133 CREB (#9198), GAPDH (#2118), phospho-Ser376 MSK1 (#9591), phospho-Ser82 Hsp27 (#2406), phospho-Thr334 MK2 (#3041), and total MK2 (#3042) were from Cell Signaling Technology (Hitchin, UK) and used at a dilution of 1 in 1000. Sheep anti-PKB alpha (S742B) and sheep anti-MSK1 (S804B) were from the Division of Signal Transduction Therapy (University of Dundee) and used at a concentration of 1 μg mL^{-1}. The phospho-Ser92 Brf1 antibody was from Abcam (Cambridge, UK, #AB79191) and used at 1:1000. Typically, 2 biological replicates were included in blotting experiments and data shown is from one replicate. The experiments shown are representative of multiple experiments.

14-3-3 pull down

BMMCs were pre-treated for 1h with DMSO or 5μM Cmp2s before stimulation with 10 ng mL^{-1} IL-33 (PeproTech). Cells were lysed in 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.27 M sucrose, 1% (vol/vol) Triton X-100, 0.1% (vol/vol) 2-mercaptoethanol, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 1 mM PMSF (triton lysis buffer). Lysates were clarified by centrifugation (13,000 rpm for 10 min at 4°C). Protein concentration was determined with Coomassie Protein Assay Reagent. 1mg of lysate per

condition were pre-cleared using Sepharose beads for 1h and then incubated for 16h at 4°C with 14-3-3-Sepharose beads[70]. Beads were pelleted by centrifugation and washed two times in triton lysis buffer with 0.15 M NaCl and once in triton lysis buffer. When indicated in the figure legends, to phosphatase treat samples, following the pulldown beads were pelleted by centrifugation and washed three times in PMP buffer (New England Biolabs, Hitchin, UK) and treated with or without 1000 units of λ phosphatase (New England Biolabs) in PMP buffer at 30°C for 30 min. 2X SDS sample buffer was added directly to beads which were then boiled for 10min to release bound proteins.

qPCR analysis

Total RNA was extracted from cells following stimulation using RNeasy kits (Qiagen, Manchester, UK), according to manufacturer's instructions. 0.5-1 µg RNA was reverse transcribed into cDNA using iScript and the resulting cDNA analysed by qPCR using a Sybr-Green based detection (Takara Bio Europe, Saint-Germain-en-Laye, France). 18s and/or GAPDH were used for normalisation and results calculated as fold stimulation relative to the unstimulated wild type cells as described[42]. Primers used are listed in Supplementary table 2.

Analysis of cytokine levels

Following stimulation of cells, the levels of TNF, IL-6, IL-10, IL-13, IL-12p40, GM-CSF, CCL3, CCL4 and CXCL2 present in the media was determined via a multiplex Luminex based method (Bioplex, BioRad, Watford, UK). For the analysis of intracellular TNF levels, BMMCs were stimulated for 4h with 10 ng mL^{-1} IL-33 in the presence of 3 µg mL^{-1} Brefeldin A and 2 µM Monensin to block cytokine secretion. Cells were then incubated with Fc Block (1 in 50 dilution, BD Biosciences) for 10 minutes, fixed and permeabilized (Fixation/Permeabilization Kit, BD Biosciences) and stained for TNF (TNF-PE, clone TN3-19.12, Biolegend, 1 in 200) and analysed by flow cytometry.

Peritoneal neutrophil recruitment model

Mice were injected intraperitoneally with 100 µL PBS, or 5 µg kg^{-1} IL-33 (PeproTech) or 2.5 mg kg^{-1} LPS (*Escherichia coli* strain O26:B6; Sigma; L2654) in 100 µL PBS. After 3 hours, the mice were sacrificed via an increasing concentration of CO_2, and the peritoneal cavity was washed using 4 mL PBS containing 1% BSA and 5 mM EDTA. Cells were pelleted by centrifugation and incubated on ice

with Fc Block (1 in 50 dilution, BD Biosciences) for 10 minutes. Cells were then stained with anti-Gr1-PerCp-Cy5.5 (1 in 800, BD Biosciences, #552093) and anti-CD11b-APC (1 in 1600, BD Biosciences #553312) for 30 min. Cells were then washed twice and analysed by flow cytometry. For analysis, live cells were gated based on forward and side scatter and neutrophils defined as CD11b Gr1 double positive cells. Gating and representative FACS plots are shown in Supplementary figure 12.

Neutrophil isolation

Mouse bone marrow-derived neutrophils were isolated from femurs and tibias as previously described[71]. Briefly, bone marrow was flushed from the bones with ice cold PBS and separated on a gradient prepared by overlaying 3 ml Histopaque 1119 (Sigma), 3 ml Histopaque 1077 (Sigma-Aldrich, Gillingham, UK) and 1 ml PBS containing the bone marrow cells. The gradient was centrifuged for 30 min at 2000rpm without brakes, and neutrophils were collected from the interface of Histopaque 1119 and Histopaque 1077.

***In vitro* chemotaxis assay**

$1x10^6$ cells BMMCs in 600 µl mast cell media in a 24-well plate were stimulated with 10 ng mL^{-1} IL-33 for 4 h or left unstimulated. ThinCert Transwell inserts with 3 µm pore size (Greiner bio-one) were allowed to equilibrate in wells for 10 min. $1x10^6$ neutrophils in 200 µl mast cell media were placed in the upper compartment of each of the transwell chambers. After incubation for 1 h at 37^oC and 5% CO_2, cells were harvested from both upper and lower chambers. GR-1^+ $CD11b^+$ were enumerated using flow cytometry by comparison with Precision Count beads (BioLegend) added to each sample. Neutrophil migration was determined as the percentage of neutrophils migrating to the lower chamber as a percentage of total neutrophils.

Statistical analysis

Data are presented as mean values +/- standard deviation unless otherwise stated. Students *t*-test (two tailed, unpaired) was performed in Excel and ANOVA testing in SigmaPlot. For post hoc analysis of ANOVA tests the Holm-Sidak method was used.

ACKNOWLEDGMENTS

This work was funded by the Medical Research Council and Arthritis Research UK. We thank Jonathan Dean (Kennedy Institute of Immunology) for bone marrow from TTP mice.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interests.

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FIGURE LEGENDS

Figure 1. p38 is required for cytokine secretion in IL-33 stimulated BMMCs

- **(a)** BMMCs were isolated from wild type mice. Where indicated, cells were pre-treated for 1h with 1 μM VX-745 before stimulation with 10 ng mL^{-1} IL-33 for the indicated times. The levels of the indicated proteins were then determined by immunoblotting.
- **(b)** As (a) but BMMCs were stimulated with 10 ng mL^{-1} IL-33 for 8 h and the levels of TNF, IL-6, IL-13, GM-CSF, CCL3 and CCL4 determined as described in the methods. Graphs represent the mean and standard deviation of results from independent cultures from 4 mice. For a comparison with the IL-33 stimulated condition, a *P*-value (two tailed students *t*-test) of less than 0.05 is indicated by *, Less than 0.01 by ** and less than 0.001 by ***.

Figure 2. Regulation of IL-33 induced cytokine production by MK2/3

(a) Wild type or MK2/3 knockout BMMCs were stimulated for the indicated times with 10 ng mL^{-1} IL-33 and the levels of total and phospho-p38, total and phospho-MK2 determined by immunoblotting. Total ERK1/2 was examined as a loading control.

(b) BMMCs from wild type or MK2/3 knockout mice were stimulated with 10 ng mL^{-1} IL-33 for 0, 4, 8 or 16 h and the levels of TNF, IL-6, IL-13, GM-CSF, CCL3 and CCL4 secreted into the media were determined. Graphs represent the mean and standard deviation of results from independent cultures from 4 wild type mice or 3 MK2/3 knockout mice. Two way ANOVA indicated a significant effect of genotype on all the cytokines tested, ($p < 0.001$, $F = 233$, 135, 102, 424, 334 or 514 for TNF, IL-6, IL-13, GM-CSF, CCL3 or CCL4 respectively). For individual time points *P*-value (post hoc Holm Sidak testing) of less than 0.05 is indicated by *, Less than 0.01 by ** and less than 0.001 by ***. N.D. indicates a condition for which values not determined.

(c) Wild type BMMCs were pre-treated with 5 μM PF-3644022, 5 μM Cmp28 or 5 μM Cmp2s for 1 h where indicated and then stimulated with 10 ng mL^{-1} IL-33 for the indicated times. The levels of the indicated proteins were determined by immunoblotting.

(d) BMMCs were pre-treated with 5 μM PF-3644022, 5 μM Cmp28 or 5 μM Cmp2s for 1h as shown and then either left unstimulated or stimulated with 10 ng mL^{-1} IL-33 for 8 h. The levels of TNF, IL-6, IL-13 and GM-CSF secreted in to the media were determined as described in the methods. Graphs represent the mean and standard deviation of results from 4 independent stimulations. For a comparison with the no inhibitor IL-33 stimulated condition, a *P* -value (post hoc Holm Sidak test after one way ANOVA) of less than 0.001 is indicated by ***.

Figure 3. IL-33 induced cytokine production is independent of TTP in mast cells.

(a) BMMCs were cultured from wild type or TTP knockout mice. Cells were stimulated for 0, 4, 8 or 16 h with 10 ng mL^{-1} IL-33 and levels TNF, IL-6, IL-13 and GM-CSF secreted into the media measured.

(b) BMMCs were cultured from wild type or TTP knockin mice in which the MK2 phosphorylation sites in TTP were mutated to alanine. Where indicated cells were pre-treated with 5 μM PF-3644022, 5 μM Cmp28 or 5 μM Cmp2s for 1 h. Cells were then either left unstimulated of stimulated with 10 ng mL^{-1} IL-33 for 8 h. TNF IL-6, IL-13 and GM-CSF secretion was then measured. In (A) and (B) graphs show the mean and standard deviation of independent cultures from 4 mice per genotype. A *P* -value (Holm Sidak test following

two way ANOVA testing) between wild type and knockout of less than 0.05 is indicated * and p < 0.001 by ***. A *P*-value for the comparison of inhibitor treated conditions and the IL-33 stimulated sample within one genotype of less than 0.001 is shown by +++.

Figure 4. Effect of MK2/3 knockout in BMMCs on IL-33 induced cytokine mRNA levels.

(a) BMMCs from wild type or MK2/3 knockout mice were stimulated with 10 ng mL^{-1} IL-33 for the indicated times. Cells were then lysed, total RNA isolated and cytokine induction determined by qPCR as described in the methods. The induction of TNF, IL-6, IL-13 and GM-CSF mRNA relative to wild types unstimulated cells is shown. Two way ANOVA indicated a significant effect of genotype on IL-6 (F=55.12, *P* < 0.001), IL-13 (F=23.27, p < 0.001) and GM-CSF (F=6.23, *P* =0.019).

(b) As (a) but primary unspliced transcript (1^{o} transcript) levels in the same cDNA samples was determined. In (A) and (B), graphs represent the mean and standard deviation of results from independent cultures from 4 wild type mice or 3 MK2/3 knockout mice. Two way ANOVA indicated a significant effect of genotype on IL-6 (F=6.92, *P* =0.014). In (a) and (b) for comparisons between genotype at individual time points, a *P*-value (post hoc Holm Sidak test following 2 way ANOVA) of less than 0.05 is indicated by *, < 0.01 by ** and < 0.001 by ***.

(c) Wild type or MK2/3 knockout BMMCs were stimulated for 4 h with 10 ng mL^{-1} in the presence of 3 μg mL^{-1} Brefeldin A and 2 μM Monensin to block cytokine secretion. Cells were then fixed and permeabilised, stained for TNF and analysed by flow cytometry.

(d) As (c) except wild type cells were pre-treated with 5 μM PF-3644022 for 1 h where indicated. In (c) and (d) Data are representative of 2 biological replicates.

Figure 5. Effect of MK2/3 knockout on cytokine mRNA stability in IL-33 stimulated BMMCs.

(a) Wild type or MK2/3 knockout BMMCs were stimulated with 10 ng mL-1 IL-33 for 1 h. 5 μg mL^{-1} Actinomycin D and 50 μM DRB then added to block transcription. Total RNA was isolated at 0, 0.5, 1 and 2h after addition of actinomycin D and DRB and the levels of TNF, IL-6, IL-13 and GM-CSF mRNA determined by qPCR. Graphs show the mean and standard deviation of independent cultures from 4 mice per genotype. Two way ANOVA showed a significant effect of genotype for TNF and GM-CSF (F=112.255, *P* < 0.001 and F=51.582 *P* < 0.001 respectively) For individual time points, in post hoc testing *P*-values between wild type and knockout of less than 0.05 are indicated * and less than 0.001 by ***.

(b) Wild type or MK2/3 KO BMMCs were stimulated with 10 ng mL^{-1} IL-33 for the indicated times. Cells were then lysed and the levels phosho-Ser92 Brf1, Brf1, p-p38, total p38 and GAPDH were determined by immunoblotting.

(c) Wild type BMMCs were incubated where indicated with 5 μM VX-745 or 5μM Cmp28. Cells were stimulated with 10 ng mL^{-1} IL-33 for the times shown and blotted for the indicated proteins.

(d) Wild type or MK2/3 knockout BMMCs were stimulated with 10 ng mL^{-1} IL-33 for the indicated times. Cells were then lysed and 14-3-3 pulldowns performed as described in the methods. Pull downs (PD) were blotted for the presence of Brf1 while lysates were blotted for phospho p38, total p38 and GAPDH.

(e) Wild type BMMCs where incubated for 1 h with DMSO or 5μM Cmp2s before stimulation with 10 ng mL^{-1} IL-33 for the indicated times. Cells were then lysed and 14-3-3 pulldowns performed as described in the methods. Pull downs (PD) were blotted for the presence of Brf1 while lysates were blotted for phospho p38, total p38 and GAPDH.

Figure 6. Inhibitors of the PI3 Kinase – Akt pathway reduce IL-33 induced cytokine induction.

(a) Wild type BMMCs were pre-treated where indicated with 1 μM PI-103, 1 μM GDC-094, 1 μM Akti 1/2 or 1 μM MK2206. Cells were then stimulated with 10 ng mL^{-1} IL-33 for 8 h or left unstimulated and the levels of TNF, IL-6, IL-13 and GM-CSF secreted into the media determined.

(b) As (a) except cells were stimulated for 1 h with 10 ng mL^{-1} IL-33. Total RNA was then extracted and the induction of TNF, IL-6, IL-13 and GM-CSF mRNA determined relative to unstimulated cells. In (a) and (b) a *P*-value of < 0.5 is indicated by * and $p < 0001$ by *** (Holm Sidak test following one way ANOVA).

(c) Wild type BMMCs were pre-treated where indicated with 1 μM PI-103, 1 μM GDC-094, or 5μM PF-3604422. Cells were then stimulated with 10 ng mL^{-1} IL-33 for 8 h or left unstimulated and the levels of TNF, IL-6, IL-13 and GM-CSF secreted into the media determined. In (a) – (c), graphs show the mean and standard deviation of independent cultures from 4 mice. A *P*-value (two tailed Students *t*-test) between the IL-33 stimulated

cells and other conditions of less than 0.05 is indicated *, less than 0.01 by ** and less than 0.001 by ***.

(d) Wild type of MK2/3 KO BMMCs were stimulated with 10 ng mL^{-1} IL-33 for the indicated times. Cells were then lysed and the levels phospho-Ser473 Akt, phospho-Thr308 Akt, total Akt, and GAPDH were determined by immunoblotting.

(e) Wild type BMMCs were incubated where indicated with 1 μM VX-745. Cells were stimulated with 10 ng mL^{-1} IL-33 for the times shown and blotted for the indicated proteins.

Figure 7. MK2/3 are required for neutrophil recruitment following i.p. injection of IL-33.

(a) Wild type, MK2/3 or MSK1/2 were injected with either PBS, 5 μg kg^{-1} IL-33 or 2 mg kg^{-1} LPS. 3 h later mice were sacrificed and the peritoneum washed to obtain cells. The % of neutrophils (CD11b+ve / Gr1+ve) in the peritoneal wash was determined by staining for CD11b and Gr1 and analyzed by flow cytometry. Graphs show average and standard deviation with crosses representing data for an individual mouse. Two way ANOVA testing between wild type and MK2/3 knockout mice indicated a significant interaction between genotype and treatment (F=19.34, $P < 0.001$). For post hoc analysis on the effect of genotype within treatment groups a *P*-value < 0.01 is indicated ** and I < 0.001by ***. The difference between MSK1/2 knockout and wild type animals treated with IL-33 was not significant ($P < 0.05$, Students *t*-test).

(b) Transwell assays were used to determine the migration of wild type neutrophils in response to wild type or MK2/3 knockout peritoneal mast cells (4 biological replicates per genotype) that were either unstimulated or treated with IL-33, as described in the methods. Blank indicated wells with neutrophils but no mast cells. Two way ANOVA testing between wild type and MK2/3 knockout mast cells indicated a significant interaction between genotype and treatment (F=12.404, $P < 0.004$). For post hoc analysis on the effect of genotype within treatment groups a *P*-value < 0.01 is indicated by ** and $P < 0.001$ by ***.

(c) Peritoneal mast cells were stimulated with 10 ng mL^{-1} IL-33 for the indicated times and the levels of CCL3, CCL4 and CXCL2 in the media determined. Data shows mean and standard deviation of 4 biological replicates per genotype. Two way ANOVA testing between wild type and MK2/3 knockout mast cells indicated a significant effect of genotype (F=160.7, $P <$ 0.001 for CCL3; F=5.26, P=0.034 for CCL2 and F=151.7, $P < 0.001$ for CXCL2). For post hoc analysis on the effect of genotype at specific times a *P*-value < 0.05 in indicated by *, < 0.01

by ** and < 0.001 by ***.